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<b>(21) International Application Number:</b> PCT/CA91/00376 <b>(22) International Filing Date:</b> 24 October 1991 (24.10.91)  <b>(30) Priority data:</b> 603,133                      25 October 1990 (25.10.90)      US  <b>(71) Applicant:</b> UNIVERSITY OF SASKATCHEWAN [CA/CA]; Saskatoon, Saskatchewan S7N 0W0 (CA).  <b>(72) Inventors:</b> REDMOND, Mark, J. ; 18 Harrington Place, Saskatoon, Saskatchewan S7K 2S3 (CA). IJAZ, Mohammed, Khalid ; Apt 304-215 Kingsmer Boulevard, Saskatoon, Saskatchewan S7J 4J6 (CA). PARKER, Michael, D. ; Apt 1706-311-6th Avenue, Saskatoon, Saskatchewan S7K 2S3 (CA).		<b>(74) Agent:</b> ADE & COMPANY; 1700-360 Main Street, Winnipeg, Manitoba R3C 3Z3 (CA).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU <sup>+</sup> , TD (OAPI patent), TG (OAPI patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ASSEMBLED VIRAL PARTICLES AND THEIR USE IN A VACCINE TO ROTAVIRAL DISEASE  <div style="text-align: center;"> </div>		
<b>(57) Abstract</b>  Assembled viral particles derived from rotavirus proteins are disclosed. The assembled particles include the inner capsid protein, VP6, in combination with either or both of the outer capsid proteins, VP4 and VP7. These assemblies can be used in vaccine compositions for the treatment and prevention of rotaviral disease.		

# + DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

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5           ASSEMBLED VIRAL PARTICLES AND THEIR USE IN A  
              VACCINE TO ROTAVIRAL DISEASE

Description

10    Technical Field

              The present invention relates generally to virus-like particles which are useful as vaccines and immunogens. In particular, the instant invention concerns assembled rotaviral structural proteins and the use of the assembly in preventing and ameliorating rotaviral infection.

Background of the Invention

              Rotaviruses cause gastrointestinal disorders and diarrhea in a wide variety of avian and mammalian species, including man. Several serotypes of rotavirus have been identified, four of which (serotypes 1 to 4) are found in humans and five of which (serotypes 3 to 7) are found in other animals. Recent studies indicate that cross protection among strains belonging to different serotypes may occur in animals including man. Ijaz et al., J Virol (1990) (In Press); Flores et al., J Clin Microbiol (1989) 27:512-518. The rotavirus genome is thought to consist of eleven segments of double-stranded RNA. The eleven genes encode the production of at least six structural proteins of the virus. In complete virus particles, these six proteins occur in a double-shelled arrangement. The outer shell or capsid is comprised of three proteins--virus protein 7 (VP7), virus protein 4

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(VP4), and a third protein which has not yet been well characterized. There are three inner shell proteins designated virus protein 1 (VP1), virus protein 2 (VP2), and virus protein 6 (VP6).

5 VP7 is the major outer shell glycoprotein with an approximate molecular mass of 38 kD in its unreduced form (as determined by SDS-PAGE) and 42 kD in its reduced form (as determined by SDS-PAGE). VP7 has approximately 325 amino acids. The amino acid sequence of several  
10 rotavirus isolates has been determined and the sequences are approximately 75 to 86% homologous. Regions of conservation among human and animal species have been reviewed by Estes, M.K. et al., Microbiol Rev (1989) 53:410-449. This protein is known to bind to host cells  
15 (Sabara, M. et al., J Virol (1985) 53:58-66). Epitope mapping of VP7 using neutralizing monoclonal antibodies has localized a neutralizing-absorption domain to a component peptide with an approximate molecular mass of 14 kD (Sabara, M. et al., supra). Synthetic peptides  
20 derived from within this 14 kD fragment have also been shown to neutralize viral infectivity (Ijaz et al., supra).

A second outer capsid protein, VP4 (formerly designated VP3), is composed of 776 amino acids and has  
25 an approximate molecular mass of 82 kD in its unreduced form and 84 kD in its reduced form. The sequence of bovine VP4 has been determined (Potter, A.A. et al., Nucl Acid Res (1987) 15:4361) as has the partial amino acid sequence for simian VP4 (Mackow, et al., Proc Natl Acad Sci USA (1988) 85:645-649. VP4 possesses  
30 hemagglutinating activity, and induces the production of neutralizing antibodies which provide heterotypic passive protection in vivo (Offit, P.A. et al., J Virol (1986)

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58:700-703). VP4 is responsible, in combination with VP7, in determining virus serotype.

Dimers of VP4 combine to form the surface spikes of rotavirus that extend distally from the rotavirus outer shell. VP4 is important in the penetration of the virus into the host cell and infectivity is increased by the cleavage of VP4 by trypsin. Trypsin enhanced infectivity is a common feature of all rotaviruses and the cleavage site for trypsin is also conserved as reviewed in Estes et al. (supra).

The inner capsid of rotavirus includes at least three proteins designated VP1, VP2 and VP6. Of interest herein is VP6 which is a 45 kD protein. Bovine VP6 appears to exist in trimeric units in both the virus particle and in infected cells, with the intersubunit linkage consisting of noncovalent interactions. These trimeric units complex further by virtue of disulfide bridges into larger units which likely represent the ring-like structures observed by several investigators using electron microscopy.

VP6 has been identified as the subgroup antigen and has also been described as the common rotavirus group antigen since some monoclonal antibodies raised against this protein react with all rotaviruses and polyclonal serum raised against a single rotavirus type can detect most other rotavirus strains. In addition to its antigenic properties, this nucleocapsid protein is extremely immunogenic and several investigators have found that the antibody raised to this protein has neutralizing ability. (See, e.g. Offit, et al., J Virol (1986) 58:700-703).

VP6 is an effective carrier protein (Redmond, M.J., et al. Mol Immunol (1990) (In Press) and VP4 is able to associate with VP6 monomeric and oligomeric

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protein units. (Redmond, M.J., et al. supra). The VP4-VP6 association has been shown to withstand harsh treatment such as boiling in SDS. Additionally, VP6 is capable of forming particles in vitro with VP2 and VP7 using a calcium dependent process (Ready, K.F.M., et al., Virology (1988) 167:269-273). The resulting assembly is immunoreactive with antibodies specific for the whole virus as well as for immunodominant sites on VP6 and VP7 and this immunoreactivity is equivalent to that of native bovine rotavirus (BRV). (Ready, K.F.M., et al., supra).

The present invention provides assembled viral particles including peptides or proteins corresponding to VP7 and/or VP4, or immunogenic regions thereof, in combination with VP6. These assembled particles are effective as vaccines and in eliciting the production of neutralizing antibodies. Such a vaccine provides an alternative to the use of a live attenuated virus vaccine.

## 20 Disclosure of the Invention

The instant invention is based on the discovery that certain viral peptides, or epitopic regions thereof, when assembled into a viral particle, are able to elicit an immune response in a subject treated therewith.

25 Vaccines including these assemblies are safer and more practical than those composed of attenuated virus.

Accordingly, in one aspect, the invention is directed to a viral particle assembly capable of eliciting an immunological response in a vertebrate subject. The viral particle assembly comprises:

- 30 (a) an inner capsid protein substantially homologous and functionally equivalent to VP6; and
- (b) one or more outer capsid proteins selected from the group consisting of (i) a protein substantially

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homologous and functionally equivalent to VP4, or a functional fragment thereof, and (ii) a protein substantially homologous and functionally equivalent to VP7.

5           In another embodiment, the present invention is directed to a viral particle assembly capable of eliciting an immunological response in a vertebrate subject wherein the viral particle assembly comprises VP6 assembled with VP4 and VP7.

10           In yet further embodiments, the invention is directed to vaccine compositions including a pharmaceutically acceptable vehicle and the viral particle assemblies described above.

15           In other embodiments, the instant invention is directed to methods of treating and preventing rotaviral disease in a vertebrate subject using the above vaccine compositions.

20           These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

#### Brief Description of the Figures

25           Figure 1 shows the nucleotide sequence and the predicted amino acid sequence of the VP6 protein of strain C486 (bovine).

30           Figure 2 compares the amino acid sequence of rotavirus VP6 derived from several strains: Bovine RF rotavirus (ROBMCP), human 1076 rotavirus (RO1HVP6), rotavirus segment 6 inner shell protein VP6 RNA (RO1S2VP6), equine H2 rotavirus (RO1VVP6H2), equine FI14 rotavirus (RO1VVP6F1), human Wa rotavirus (RO2SEG6), porcine Gottfried rotavirus (RO1PVP6), porcine group C rotavirus (PRVVP6), and simian SA11 rotavirus (ROTG6A).

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Figure 3 compares the amino acid sequence of rotavirus VP4 derived from several strains: K8, KU, DS1, M37, ST3, SA11, and RRV.

5       Figure 4 shows the nucleotide sequence and corresponding amino acid sequence of the VP4 protein from strain C486 (bovine).

Figure 5 depicts the nucleotide sequence and corresponding amino acid sequence of the VP7 protein from strain C486 (bovine).

10       Figure 6 compares the amino acid sequence of rotavirus VP7 derived from several strains: simian 11 rotavirus (ROTV7), rhesus rotavirus (RORVP7), porcine OSU rotavirus (PRVOSUVP7), human rotavirus (ROHVP7A), porcine Gottfried rotavirus (PRVPRVP7G), bovine uk  
15       rotavirus (ROB7), and porcine major C rotavirus (PRVPRVVP7).

Figure 7 is a graphic representation of the production of in vitro assembled rotavirus particles.

20       Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, protein chemistry, molecular biology, microbiology and recombinant DNA technology,  
25       which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Handbook of Experimental Immunology, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications); Methods in Enzymology (S. Colowick and N.  
30       Kaplan eds., Academic Press, Inc.); Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual 2d Edition (Cold Spring Harbor Laboratory Press, 1989); Oligonucleotide Synthesis (M.J. Gait ed., 1984); and DNA Cloning, Volumes I and II (D.N. Glover, ed., 1985).



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All patents, patent applications, and publications mentioned herein, whether supra or infra, are hereby incorporated by reference.

5     A.           Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By "VP6" is meant the art-recognized major  
10   viral protein of the inner capsid from any species or strain within the family Reoviridae. See, e.g., Kapikian, et al., in Virology (B.N. Fields et al., eds., 1988). Examples of rotavirus strains from which the VP6 protein can be isolated and employed in the present  
15   invention include, but are not limited to, Simian SA11, human D rotavirus, bovine UK rotavirus, human Wa or W rotavirus, human DS1 rotavirus, rhesus rotavirus, the "O" agent, bovine NCDV rotavirus, human S2 rotavirus, human KUN rotavirus, human 390 rotavirus, human P rotavirus,  
20   human M rotavirus, human Walk 57/14 rotavirus, human Mo rotavirus, human Ito rotavirus, human Nemoto rotavirus, human YO rotavirus, human McM2 rotavirus, rhesus monkey MMU18006 rotavirus, canine CU1 rotavirus, feline Taka rotavirus, equine H2 rotavirus, human St. Thomas No. 3  
25   and No. 4 rotaviruses, human Hosokawa rotavirus, human Hochi rotavirus, porcine SB2 rotavirus, porcine Gottfried rotavirus, porcine SB1A rotavirus, porcine OSU rotavirus, equine H1 rotavirus, chicken Ch.2 rotavirus, turkey Ty.1 rotavirus, bovine C486 rotavirus, and strains derived  
30   therefrom. Thus VP6 for use in the present invention includes VP6 from any rotavirus strain, whether from subgroup I, subgroup II, or any as yet unidentified subgroup, as well as from any of the serotypes 1-7, as well as any as yet unidentified serotypes.

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The VP6 protein comprises an amino acid sequence of rotavirus which is unique to the class, or any member of the class, of VP6 polypeptides. A representative nucleotide sequence and the deduced amino acid sequence of bovine recombinant (BR) VP6 strain C486 is shown in Figure 1. Figure 2 shows the amino acid sequence of several different strains of rotavirus. As can be seen, extensive homology is present between the depicted rotavirus strains. Other VP6 nucleotide and amino acid sequences will find use in the instant invention, the depicted sequences only being representative of already known VP6 sequences.

By "VP4" is meant an art-recognized viral protein of the outer capsid from any species or strain within the family Reoviridae. Examples of rotavirus strains from which the VP4 protein can be isolated and employed in the present invention include, but are not limited to, those described above with reference to VP6.

VP4 (formerly designated VP3), is composed of 776 amino acids. The amino acid sequence of VP4 derived from strains K8, KU, DS1, M37, ST3, SA11, and RRV is shown in Figure 3. The nucleotide sequence and deduced amino acid sequence of VP4 from strain C486 (bovine) is shown in Figure 4. Again, other sequences will find use herein.

By "VP7" is meant the art-recognized major viral protein of the outer capsid from any species or strain within the family Reoviridae. Examples of rotavirus strains from which the VP7 protein can be isolated and employed in the present invention include, but are not limited to, those described above with reference to VP6.

VP7 is composed of approximately 325 amino acids and the nucleotide sequence and deduced amino acid

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sequence of VP7 from strain C486 (bovine) is shown in Figure 5. Arias et al., J Virol (1984) 50:657-661 describes the nucleotide sequence of VP7 from simian SA11. Figure 6 depicts the amino acid sequences of several rotavirus strains. VP7 shows serotype restricted homology. The above sequences are meant to be representative and nonlimiting. Therefore, other functional sequences may also be employed with the present invention.

10           Two DNA or protein sequences are "substantially homologous" when at least about 85% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. DNA sequences that are substantially  
15 homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., supra.

20           The term "functionally equivalent" refers to sequences of an analog of an outer or inner capsid rotavirus protein which define a chain that will produce a protein that elicits an immunological response equivalent to that elicited by the native sequence.  
25 Thus, the rotavirus proteins utilized herein need have the identical amino acid sequence of the native proteins.

          A "functional fragment" of a rotavirus protein is a fragment with the capability of raising an immunological response equivalent to that elicited by the  
30 full sequence. It has been demonstrated that the distal end of VP4 is involved in the initial attachment of the virion to the cell, since infection may be blocked by monoclonal antibodies to this region. Furthermore, the enzyme trypsin enhances virus infectivity. This

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enhancement appears to act after adsorption, since trypsin does not affect the efficiency or rate of virus attachment to cells but does increase the levels of uncoated particles found in cells. The molecular  
5 mechanism for trypsin enhanced infectivity occurs via the cleavage of the VP4 protein into two fragments with approximate molecular weights of 28 kDa and 60 kDa, respectively. Therefore, the trypsin cleavage site of VP4 is important in rotavirus replication. Thus,  
10 fragments consisting of an amino acid sequence substantially homologous to at least the first 255 N-terminal amino acids, as depicted in Figure 4, and which include the trypsin cleavage site, will also find use in the instant invention so long as these fragments are  
15 capable of raising an immunological response as defined above.

"A viral particle assembly" refers to an association between outer and inner capsid proteins of rotavirus, or proteins substantially homologous and  
20 functionally equivalent thereto, or functional fragments thereof. The particles can be assembled in vitro, as described more fully below, to resemble double shelled rotavirus.

The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. The term is also used interchangeably with  
25 "antigenic determinant" or "antigenic determinant site."

An "immunological response" to a composition or vaccine is the development in a vertebrate subject of a  
30 cellular and/or antibody mediated immune response to the composition or vaccine of interest. Usually, such a response consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or

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antigens included in the composition or vaccine of interest.

An "immunogenic protein" is a protein which elicits an immunological response in a subject to which it is administered.

The terms "polypeptide" and "protein" are used interchangeably herein and are used in their broadest sense, i.e., to denote any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the terms encompass oligopeptides, protein fragments, analogs, mutants, fusion proteins and the like.

"Native" proteins or polypeptides refer to proteins or polypeptides recovered from rotavirus or from rotavirus infected cells. Thus the term includes naturally occurring rotavirus proteins and fragments thereof. "Non-native" polypeptides refer to polypeptides that have been produced by recombinant DNA methods or by direct synthesis. "Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide.

A "replicon" is any genetic element (e.g., a plasmid, a chromosome, a virus) that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

A "vector" is a replicon in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment. An "expression vector" refers to a vector capable of autonomous replication or integration and contains control sequences which direct the transcription and translation of the desired DNA sequence in an appropriate host.

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A "coding sequence" is a polynucleotide sequence which is transcribed and/or translated into a polypeptide.

5 A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase and initiating transcription of a downstream (i.e., in the 3' direction) coding sequence.

10 A coding sequence is "under the control" of the promoter sequence in a cell when transcription of the coding sequence results from the binding of RNA polymerase to the promoter sequence; translation of the resulting mRNA then results in the polypeptide encoded within the coding sequence.

15 "Operably linked" refers to a juxtaposition wherein the components are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence.

20 "Control sequences" refers to those sequences which control the transcription and/or translation of the coding sequence(s); these may include, but are not limited to, promoter sequences, transcriptional initiation and termination sequences, and translational initiation and termination sequences. In addition,  
25 "control sequences" refers to sequences which control the processing of the polypeptide encoded within the coding sequence; these may include, but are not limited to sequences controlling secretion, protease cleavage, and glycosylation of the polypeptide.

30 A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this

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signal peptide is clipped off by the host cell before the protein leaves the cell.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or

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naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

A composition containing A is "substantially free of" B when at least about 85% by weight of the total of A + B in the composition is A. Preferably, A comprises at least about 90% by weight of the total of A + B in the composition, more preferably at least about 95%, or even 99% by weight.

A "vaccine composition," according to the present invention, is an otherwise conventional vaccine formulation employing either the viral particle assemblies alone or in combination with one or more unassembled purified viral proteins or with cell lysates having one or more of the individual outer and/or inner capsid proteins. Particularly useful is the addition of a crude cell lysate containing VP4 to the instant vaccine compositions. The preparation of vaccines containing the above active ingredients is well understood in the art. Typically, vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposomes. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccine. The vaccines are conventionally administered parenterally, by injection, for example,



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either subcutaneously or intramuscularly. Injectable vaccine formulations will contain an effective amount of the active ingredient, the exact amount being readily determined by one skilled in the art. The active  
5 ingredient can range from about 0.01% to about 95% (w/w) of the injectable composition, or even higher or lower if appropriate.

Additional vaccine formulations which are suitable for other modes of administration include  
10 suppositories and, in some cases, oral formulation. For suppositories, the vaccine composition will include traditional binders and carriers, such as polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in  
15 the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium, stearate, sodium saccharin cellulose,  
20 magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 10% to about 95% of the active ingredient, preferably about 25%  
25 to about 70%.

Furthermore, the viral particles may be formulated into vaccine compositions in either neutral or salt forms. If salts are used, the final preparation will typically contain less than 0.15M salt.  
30 Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric,

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mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylaminoethanol, histidine, procaine, and the like.

By "treating" is meant curing or ameliorating a subject that has contracted a rotaviral infection.

"Preventing" rotaviral disease means preventing the occurrence of the infection, or tempering the severity of the infection if it is contracted subsequent to the administration of the instant compositions.

The vaccine composition of the present invention may be administered in a manner compatible with the dosage formulation, and in such amounts as will be therapeutically effective and immunogenic. A "therapeutically effective amount" of a vaccine composition is a dose sufficient to either prevent or treat rotaviral infection in a subject to which the composition is administered. The dosages of the viral particle assemblies which can treat or prevent rotaviral infection can be determined in view of this disclosure by one of ordinary skill in the art by running routine trials with appropriate controls. Comparison of the appropriate treatment groups to the controls will indicate whether a particular dosage is effective in preventing or treating a disease used in a controlled challenge. In general, effective dosage will vary depending on the mode of administration. For example, in the case of an intramuscular injection, generally from 0.001  $\mu\text{g/kg}$  to 10  $\mu\text{g/kg}$  will find use in the instant invention.

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B. General Methods

Central to the instant invention is the discovery that assembled viral particles comprising rotavirus inner and outer capsid proteins, are able to elicit an immune response in a subject to which they are administered. The viral particles are assemblies of inner and outer capsid proteins of rotavirus. Particularly useful is a viral particle assembly including the inner capsid protein, VP6, with either or both of the outer capsid proteins, VP4 and VP7. VP6 appears to act as a "carrier" for the two outer capsid proteins, as described more fully below. These assemblies can be used alone, or in combination with unassembled, outer capsid proteins, provided as purified or partially purified proteins, or crude cell lysates, in a vaccine composition for the treatment and/or prevention of rotaviral infection.

The inner and outer capsid proteins for use in the viral particles of the instant invention can be prepared by any of several methods. First, the individual proteins can be isolated by successive degradation of purified virus with EDTA and either calcium chloride ( $\text{CaCl}_2$ ) or lithium chloride ( $\text{LiCl}$ ) treatment by standard techniques. See, e.g., Almeida et al., J Med Virol (1979) 4:269-277; Bican et al., J Virol (1982) 43:1113-1117; Gorziglia et al., J Gen Virol (1985) 66:1889-1900; Ready et al., Virology (1987) 157:189-198. Alternatively, the viral proteins can be produced by recombinant DNA techniques, which are fully explained in the literature. See, e.g., Sambrook et al., supra; and DNA Cloning, supra.

DNA coding sequences encoding the viral polypeptides can be derived from the particular mRNA. See, e.g., Estes et al., supra; Both et al., J Virol

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(1984) 51:97-101; Cohen et al., Virology (1984) 138:178-182. Alternatively, a DNA sequence encoding the particular viral protein can be prepared synthetically rather than cloned. The DNA sequence can be designed  
5 with the appropriate codons for the viral protein amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods  
10 and assembled into a complete coding sequence. See, e.g., Edge, Nature (1981) 292:756; Nambair et al., Science (1984) 223:1299; Jay et al., J Biol Chem (1984) 259:6311.

Once a coding sequence for the viral protein  
15 has been prepared or isolated, it can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host  
20 cells which they can transform (in parenthesis) include the bacteriophage lambda (E. coli), pBR322 (E.coli), pACYC177 (E. coli), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria),  
25 pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), YIp5 (Saccharomyces), YCp19 (Saccharomyces) and bovine papilloma virus (mammalian cells). See generally, DNA Cloning: Vols. I & II, supra; and Sambrook et al.,  
30 supra.

The coding sequence for the viral protein can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control"

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elements), so that the DNA sequence encoding the viral protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain  
5 a signal peptide or leader sequence. In bacteria, for example, the viral protein is preferably made by the expression of a coding sequence containing a leader sequence which is removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos.  
10 4,431,739; 4,425,437; 4,338,397.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the  
15 control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). The control sequences may be ligated to the coding  
20 sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

25 A number of procaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also UK Patent Applications GB 2,121,054; GB 2,008,123; GB  
30 2,007,675; and European Patent Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; and 96,491.

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Particularly useful for expression of the rotaviral genes of the instant invention are insect cells and vectors suitable for use in these cells. Such systems are known in the art, and include, for example, insect expression transfer vectors constructed from the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV). Such expression vectors typically use the strong viral polyhedrin gene promoter to control expression of heterologous genes. Methods for the introduction of heterologous DNA into the desired site in the baculovirus are known in the art. (See, e.g. Smith, et al., Mol and Cell Biol (1983) 3:2156-2165; and Luckow and Summers Virology (1989) 17:31. Insertion can be, for example, into a gene such as the polyhedrin gene, by homologous recombination. Insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene. Sequences encoding signal peptides can also be used in these expression systems since these peptides are recognized by insect cells and will cause the secretion of the expressed product into the expression medium.

Depending on the expression system and host selected, the viral protein is produced by growing host cells transformed by an expression vector described above under conditions whereby the protein is expressed. The protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the protein can be purified directly from cell-free media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

Purified VP6 protein exhibits structural polymorphism. Specifically, hexamers and small hexagonal

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lattices are present in many of the samples. Tubular particles form between about pH 5.0 and about pH 9.0, and are moderately stable to changes in temperature and ionic strength. The formation of these particles is fully reversible. Spherical particles reassembling single-shelled virus can be formed at about pH 4.0. A novel structure, in the form of sheets, composed of small-hole lattice, is formed in samples shifted from about pH 6.0 to about pH 4.0. These results demonstrate the importance of VP6 and of protein-protein interactions for rotavirus assembly.

Such protein-protein interactions are likely involved in the observed phenomenon that certain peptides can bind to VP6 in its monomeric form or to various oligomeric structures formed from VP6 monomers, such as in vitro assembled tubes and spheres. The attachment is mediated by a specific binding site(s) within VP6.

After the individual outer and inner capsid proteins have been either isolated, synthesized, or recombinantly produced, these proteins are assembled into viral particles using a calcium dependent process described in the examples and in Ready, K.F.M., et al., Virology (1988) 167:269-273. Figure 7 depicts a representative scheme for the production of in vitro assembled rotavirus particles.

As explained above, the viral particles can be administered in vaccine compositions in combination with purified, unassembled outer capsid proteins, or with cell extracts including one or more of the individual outer capsid proteins. These cell extracts are prepared by techniques well known in the art. For example, cell extracts can be prepared by cultivating rotavirus in African Green Monkey MA104 cells, harvesting the cells and media together and removing the cells by centri-

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fugation. The resulting supernatant includes rotavirus proteins. The proteins can be further purified using density gradient ultracentrifugation. The virus pellet is then subjected to treatment with  $\text{CaCl}_2$  and  $\text{LiCl}_2$ , as described in Ready, K.F.M. and Sabara, M. Virology (1987) 157:189-198, to obtain the individual viral particles. VP4 is present in very small amounts in such preparations. Furthermore, small amounts of VP4 are often present in the VP7 fractions.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

15

#### Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strains was made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR §1.14 and 35 USC §122. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, lose its plasmid, it will be



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replaced with a viable culture(s) of the same taxonomic description.

	<u>Strain</u>	<u>Deposit Date</u>	<u>ATCC No.</u>
5	pAC373BRV6 (in <u>E. coli</u> )	31 August 1987	40362
	BVLVP4 (in <u>A. californica</u> )	24 October 1990	
10	BYVP7 (in <u>A. californica</u> )	24 October 1990	

C. Experimental

Materials and Methods

15

Enzymes were purchased from commercial sources, and used according to the manufacturers' directions. Radionucleotides and nitrocellulose filters were also purchased from commercial sources.

20

In the cloning of DNA fragments, except where noted, all DNA manipulations were done according to standard procedures. See, Sambrook et al., supra. Restriction enzymes, T<sub>4</sub> DNA ligase, E. coli, DNA polymerase I, Klenow fragment, and other biological reagents were purchased from commercial suppliers and used according to the manufacturers' directions. Double-stranded DNA fragments were separated on agarose gels.

25

Animals:

30

Rotavirus-free mice (CD1) were purchased from Harlan Sprague Dawley Inc., (Indianapolis, IN). The age of the mice at the time of purchase was approximately 6 weeks. The mice weighed between 25 and 30 grams. All mice were found to be seronegative for antibodies to rotavirus using an ELISA assay (Ijaz, M.K. et al. Exp Mol

35

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Path (1989) 51:186-204). The animals were housed in isolation units throughout the experiment.

Cells and Virus:

5 MA104 cells (African green monkey) were  
cultured in Eagle's minimal essential media (MEM)  
supplemented with 10% fetal bovine serum (FBS) (Gibco  
Laboratories, Grand Island, NY). Bovine rotavirus  
isolate C486 was cultured from the feces of diarrheic  
10 calves by a method described previously (Babiuk, L.A. et  
al., J Clin Microbiol (1977) 6:610-617). Rotavirus C486  
is publicly available from the ATCC, Rockville, MD  
(accession no. VR-917). Simian rotavirus strain SA11  
(serotype 3) was obtained from Dr. H. Malherbe (San  
15 Antonio, TX) and human rotavirus strain DS1 (serotype 2)  
and human rotavirus strain Wa (serotype 1) were obtained  
from Dr. H. Greenberg (Stanford University, CA).

These viruses were propagated in confluent  
MA104 cells in the presence of 1  $\mu$ g of trypsin (Difco  
20 Laboratories, Detroit, MI) per ml in the absence of FBS.  
Cells and supernatant were harvested together and cells  
were removed by centrifugation at 500g for 20 min. Virus  
was concentrated from the clarified supernatant fluids by  
pelleting through a 40% sucrose-cushion at 100,000 g for  
25 2½ hr at 15°C. The virus pellet was resuspended in  
double distilled water and the amount of virus protein  
was estimated spectrophotometrically as described  
previously (Ijaz, M.K. et al., Antiviral Res (1987)  
8:283-298). The resuspended virus was stored at -70°C.  
30 Individual inner and outer capsid proteins were isolated  
from the preparation as described in the examples below.

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Plaque Assay:

A plaque assay for the quantitation of infectious rotavirus was performed according to the method described previously (Aha, P.M. and Sabara, M.I. J Virol (1990) 28:25-32). Briefly, 12 well tissue culture plates (NUNC) containing confluent monolayers of MA104 cells, were washed twice with MEM (without FBS). Serial tenfold dilutions of each rotavirus isolate were prepared in MEM containing trypsin (Difco Laboratories, Detroit, MI) to a final concentration of 10  $\mu\text{g/ml}$ . Following adsorption of the virus at 37°C for 1 hr, the inoculum was aspirated, cells were washed with MEM and overlaid with Dulbecco's Modified Eagle Medium (DMEM), containing 4% Sephadex G-75 beads (Pharmacia). The plates were incubated for 2 days at 37°C, the overlay was aspirated and plates were stained with 0.5% crystal violet/80% methanol/PBS, washed and plaques enumerated.

ELISA Procedure:

The ELISA was a modification of a previously described procedure (Sabara, M. et al., J Virol (1985) 53:58-66). All incubations were performed at room temperature (20°C) for 1 hr unless stated otherwise. Polystyrene, 96-well Immulon 2 plates (Dynatech Labs Inc., Alexandria, VA) were sensitized for each of the assay systems and used as follows

For the detection of protein specific antibody, plates were coated overnight with the respective protein (5 picomoles/well) diluted in 0.05M carbonate bicarbonate buffer at pH 9.6. Unabsorbed protein was removed by extensive washing with distilled water. The uncoated sites on the plate were blocked by overnight treatment with 3% horse serum in 0.01M PAS and then washed with double distilled H<sub>2</sub>O. The plated antigen was overlaid

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with 75  $\mu$ l of mouse antiserum/well in 0.01M PAS containing 1% horse serum and 0.05% Tween 20. Incubation was carried out for 2 hr at room temperature after which time the unbound antibody was removed by washing with 0.01M PAS containing 0.05% Tween 20 (PBST). A 1/5000 dilution (in PBST plus 1% horse serum) of biotinylated-goat anti-mouse serum (Zymed Laboratories Inc., San Francisco, CA) was then added per well and incubated for one hr at room temperature. After washing in PBST, plates were incubated for one hr with 75  $\mu$ l of streptavidin horseradish peroxidase conjugate diluted in PBST and 1% horse serum. After washing with PBST, the substrate (2,2'-Azino-di-[3 ethyl-benzthiazoline sulfonate], ABTS, Boehringer-Mannheim) was added. The color development was stopped after 10 min by the addition of 10% SDS. The optical density of the wells was determined at 405 nm by an ELISA reader (BioRad Laboratories, Richmond, CA). Titers were expressed as a reciprocal of the highest dilution with an OD of  $>2$  SD over mean background levels.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Viral proteins were separated by SDS-PAGE under both reducing and non-reducing conditions according to the procedure described by Laemmli (Laemmli, U.K. Nature (1970) 227:680-685). Virus samples were resuspended in electrophoresis sample buffer (0.337M Tris pH 6.8, 6% SDS, 30% glycerol, 0.03% bromophenol blue) for running under nonreducing conditions and included 3.75% mercapto-ethanol (BME) for reducing conditions. The samples were boiled for 5 to 10 min and analyzed following electrophoresis on a 10% polyacrylamide resolving gel with a 3% stacking gel.

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Western Blotting of Rotavirus Proteins:

Protein-specific antibodies were detected by the Western blotting technique described by Towbin et al. (Towbin, H. et al., Proc Natl Acad Sci USA (1979) 76:4350-4354). Viral proteins separated on a 10% polyacrylamide gel, were transferred to nitrocellulose paper (0.45  $\mu$ m) (BioRad Laboratories) by electroblotting at 100 volts for one hr in a buffer containing 20 mM Tris-190 mM glycine-20% methanol. Replica nitrocellulose strips were stained with amido black to determine the efficiency of protein transfer.

After transfer, reaction of viral protein with serum samples was determined as described previously (Braun, D.K. et al., J Virol (1983) 46:103-112). Non-specific reactions were blocked with 3% bovine serum albumin (BSA) in 0.01M TBS. After washing with TBST, the reaction was developed with protein A gold (BioRad Laboratories) for one hr. Following development, the protein bands were intensified by silver enhancement (Janssen Biotech, N.V., Belgium).

Example 1Isolation of Viral Proteins25      A. Isolation of Native VP6

The VP6 viral protein was isolated from the purified virus suspension (described above) by successive degradation of purified virus with EDTA and either  $\text{CaCl}_2$  or  $\text{LiCl}$ , as follows. Outer capsid proteins were removed by incubating virus (3 mg/ml) in 50 mM EDTA, 0.01M Tris-HCl, pH 7.4 at 4°C for 30 min. Subviral particles were recovered by ultracentrifugation (100,000 xg, 2-3 hr, 4°C) and resuspended in 0.01M Tris-HCl, pH 7.4 or 0.01M sodium borate, pH 9.0. They were then treated with

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either 1.5M CaCl<sub>2</sub> with 0.01M Tris-HCl, pH 7.4 at 20°C for 20-30 min, or were frozen in 2M LiCl, 0.01M sodium borate, pH 9.0 at -70°C for 4 days. Cores and undegraded particles were separated from solubilized protein by ultracentrifugation. EDTA and salts were removed by extensive dialysis at 4°C against 0.01M Tris-HCl, pH 7.4, unless otherwise indicated. The purity of the samples was examined by polyacrylamide gel electrophoresis (PAGE) as described above.

10

#### B. Isolation of Native VP4

The limited number of copies of VP4 protein per virus particle makes the purification of large amounts of this protein difficult. However, VP4 is found in the supernatant obtained after ultracentrifugation following treatment of the subviral particles with 1.5M CaCl<sub>2</sub> or 2M LiCl treatment of intact virus particles, as described in the isolation of native VP6. VP4 can also be purified from this pellet by e.g. HPLC, affinity chromatography, ion-exchange chromatography, etc.

20

#### C. Isolation of Native VP7

As with VP4, VP7 is also found in the supernatants described above but in larger amounts. See, Ready, K.F.M., et al. Virology (1988) 167:269-273. VP7 can be further purified using HPLC, affinity chromatography, ion-exchange chromatography, etc.

25

### Example 2

30

#### Production of Recombinant Viral Protein

##### A. Production of Recombinant VP6

The construction of recombinant Autographa californica nuclear polyhedrosis virus (AcNPV) containing gene 6 from bovine rotavirus (BRV) and assembly of VP6

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particles following infection of *spodoptera frugiperda* (SF9) cells has been described previously (Redmond, M.J. et al., Mol Immunol, In Press. Briefly, genomic RNA extracted from purified bovine rotavirus strain C486 was used to produce cDNA. The cDNA was ligated into the Pst I site of pBR322 and used to transform *E. coli* strain DH1. The resulting colonies were probed with radiolabeled cDNA prepared from purified genomic RNA segment 6 as template.

10           Clone pR6-42 which contained a complete copy of the gene 6 RNA, was partially digested with Aha III which removed seven 5' noncoding nucleotides as well as the oligo-dC tails added during cDNA cloning. A Bam HI linker was then added.

15           The 3' oligo-dC tail and noncoding region were removed by digestion with Acc I which removes 56 noncoding nucleotides from the VP6 gene. A Bam HI linker was then added. The gene 6 cDNA was then ligated into the Bam HI site of the baculovirus transfer vector pAc373. This vector was designated pAc373BRV6 (ATCC no. 20 40362). Integration of the rotavirus gene into the genome of *A. californica* was then carried out by homologous recombination in *S. frugiperda* (SF9) cells as outlined by Summers, M.D. and Smith, G.E., Texas Agricultural Station Bulletin 1555:26-27. Recombinants 25 were identified by plaque hybridization, as described above, using radiolabeled cDNA prepared from purified genomic RNA segment 6. Recombinants were plaque purified and analyzed for expression of recombinant gene 6 30 produced proteins by SDS-PAGE analysis and Western blotting using the methods described above.

The recombinant virus containing gene 6 was used to infect SF9 cells. Following incubation for 72 hr at 27C, the cells were lysed in a 2 ml  $\text{NaHCO}_3$  buffer (pH

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7.5) containing 0.05% triton X-100 and 0.2 trypsin inhibitor units per ml. Cellular debris was removed by centrifugation at 1500g. The supernatant was dialyzed against 0.1M glycine buffer (pH 3.0) for 24 hr. This  
5 dissociates VP6 aggregates into monomers. The dialysis solution was exchanged for 0.01M citrate buffer (pH 4.0) and dialysis continued for 24 hr, during which time spheres of VP6 formed. This dialysis buffer was exchanged for 0.01M Tris-HCl (pH 7.4) + 1 mM sodium  
10 azide. Dialysis was then continued overnight at 4°C. Nonaggregated material was removed by ultracentrifugation using 300,000 dalton molecular weight cutoff filters. The quality of the VP6 spheres produced by this method was determined by electromicroscopy and purity confirmed  
15 by SDS-PAGE.

#### B. Production of Recombinant VP4

VP4 was produced recombinantly as described for VP6 except that site directed mutagenesis was used to  
20 insert a Bam HI restriction site immediately upstream of the translational initiation codon of the cDNA including gene 4 (the gene encoding VP4), resulting in the deletion of nine nucleotides normally present preceding the VP4 initiation codon. The 3' end of the gene was left  
25 unmodified.

The modified gene 4 was ligated into transfer vector pVL941. [DEPOSIT NUMBER] Integration of gene 4 into the A. californica genome was carried out as above to render the expression vector BVLVP4.

30 Crude detergent lysates of the VP4-baculovirus infected SF9 cells were used as starting material in the production of viral particles as described below. The interaction between VP4 and VP6 is analogous to an affinity purification step. Thus, the VP6-VP4 complex



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can be separated from unbound VP4 and other cellular proteins by ultracentrifugation over sucrose gradients.

### C. Production of Recombinant VP7

5 cDNA including a full length copy of the gene for VP7 (gene 8) was cloned and identified as described above. A clone (clone pr8-G) which contained the full length cDNA copy of the VP7 gene was digested with Aha III and Sph I which removed 7 and 27 nucleotides,  
10 respectively, from the 5' and 3' ends of the gene. Bam HI linkers were then added to both ends of the DNA.

Gene 8 was ligated into the Bam HI site of pACYM1 and integration of gene 8 into the genome of A. californica proceeded as described for VP6 to yield  
15 expression vector BYVP7. Recombinants were identified, plaque purified and assayed for expression as described above.

VP7-baculovirus infected SF9 cells secrete VP7 directly into the growth medium. VP7 is essentially  
20 affinity purified therefrom by the formation of double shelled (VP6-VP7) particles, in the presence of 50mM  $\text{CaCl}_2$ . This separates the VP7 from the other components present in the growth medium. The final purification step is ultracentrifugation over sucrose gradients.

25

### Example 3

#### Assembly of Viral Particles

VP7 and VP4 protein concentrations in growth media and infected cell lysates were estimated on SDS-  
30 PGE gels stained with Coomassie Blue, and western blots probed with VP7 and VP4 specific antisera. A standard of native BRV was used at a predetermined concentration. The concentrations of proteins used give the following ratios to VP6

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VP6-VP4. 1:10 w/w

VP6-VP7. 1:10 w/w

The starting material for all particle preparations was 10 µg of VP6. To prepare VP6-VP4 particles, 100 µg of VP4 protein in a detergent lysate of SF9 cells (described in Example 2B) dialyzed in 10 mM Tris + 50 mM CaCl<sub>2</sub> pH 8 overnight at 4°C was mixed for 2 hr. at 37° with VP6. If used as a vaccine, the preparation was centrifuged at 100,000 g. for 2 hr. through a 40% sucrose gradient.

To prepare VP6-4-7 particles, the VP6-4 particles prepared above were added to 100 µg of VP7. The VP7 preparation consisted of media from cell culture that had been dialyzed overnight into 0.1M Tris-HCl pH 8.0 containing 50 mM CaCl<sub>2</sub>.

The VP6-4+7 mixture was then dialyzed for 3-4 days against 0.1M Tris-HCl pH 8.0 containing 50 mM CaCl<sub>2</sub>. The particles were purified over sucrose as described previously.

To prepare VP6-VP7, 10 µg of VP6 spheres were mixed with 100 µg of VP7 contained in a preparation obtained by dialyzing tissue culture growth medium, from VP7-baculovirus infected SF9 cells, against 0.1M Tris-HCl pH 8.0 containing 50 mM CaCl<sub>2</sub>. Dialysis was continued for 3-4 days against the same buffer after the addition of VP6. VP6-7 particles, when required, were purified by ultracentrifugation. In order to produce VP6-7-4 particles, cell lysates containing VP4 equivalent to 100 µg of purified virus were mixed for 2 hrs. at 37°C, with VP6-7, then ultracentrifuged through 40% Sucrose for 2 hrs. at 100,000 G. After this time, particles were purified by centrifugation as described above.

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Example 4Protective Capacity of the Assembled  
Viral Proteins

Vaccine compositions used in this example were  
5 as follows: For the primary immunizing dose, each  
immunogen (Table 1) was emulsified with Freund's complete  
adjuvant. For the second and third immunizations, each  
immunogen was emulsified with Freund's incomplete  
adjuvant. Equal volumes of the immunogen and the  
10 adjuvant were used.

Thirteen groups of mice were immunized  
intramuscularly with the preparations outlined in Table  
1. Each mouse was immunized three times before and after  
breeding. The first immunization was given when the mice  
15 were seven weeks old and was followed by the second and  
third vaccinations at two week intervals. Litters were  
born when the mice were 12 to 14 weeks old.

Following birth, the mouse pups were allowed to  
suckle their dams and were challenged at 7 days of age  
20 with one of four rotavirus isolates. These isolates were  
bovine rotavirus strain C486 (serotype 6), simian  
rotavirus strain SA11 (serotype 3), human rotavirus  
strain DS1 (serotype 1) and Wa (serotype 2). The SA11  
isolate was obtained from Dr. H. Malherbe (San Antonio,  
25 Texas) and the strain Wa and DS1 isolates were obtained  
from Dr. H. Greenberg (Stanford University, California).  
The strain C486 which was a local isolate adapted to grow  
in MA104 cells (Babiuk, L.A. et al., J Clin Microbiol  
(1977) 6:610-617). These viruses were grown in MA104  
30 cells, harvested and concentrated for challenge by the  
method described previously (Ijaz et al., Antiviral Res  
(1987) 8:283-298. The challenge dose for each isolate  
was approximately  $10^4$  PFU/mouse suspended in MEM in 100  
 $\mu$ l volume. For challenge, the virus preparations were

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administered by intubation of the stomach with a soft flexible plastic feeding tube. Trypan blue dye (GIBCO) was used as a marker to assess the accuracy of intubation.

5                   The appearance of diarrhea was scored clinically up to 72 hr post-challenge using clinical scores as follows:

                  (-) no sign of diarrhea in live mice, or on necropsy;

10                   (+) no external signs of diarrhea but semi-liquid colon contents at autopsy;

                  (++) fluid was apparent on palpation of the abdomen and the colon was filled with liquid feces and gas;

15                   (+++) the external anal region was soiled with feces and intestinal fluid was present on palpation and;

                  (++++) liquid feces present around the anal region and on palpation of the abdomen intestinal fluid was present and oozed from the anus, severe dehydration,  
20                   internal liquid content in colon and caecum and distention due to accumulation of gas.

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Table 1  
HOMOLOGOUS PROTECTION OF NEONATAL MICE SUCKLING ON DAMS  
IMMUNIZED WITH RECOMBINANT ROTAVIRUS PROTEINS

Group	Antigen	Serum Titre	Wa	Clinical Score			PRN
				DS-1	SA-11	BRV	
1	Sentinel	-	++++	++++	++++	++++	
2	Placebo	-	++++	++++	++++	++++	
3	BRV	1,540,830	0	0	0	0	<40
4	VP6	5,235	++	++	++	++	>1,280
5	VP7	6,105	ND	ND	ND	+	<40
6	VP4	53,850	0	0	+	0	<40
7	VP6-VP4	31,150	0	0	+	0	780
8	VP6-VP7	252,520	0	0	+	0	1,000
9	VP6-VP4 plus VP6-VP7 (mixture)	64,805	0	0	++	0	<40
10	VP6-VP4-VP7	35,235	ND	ND	ND	0	740
11	VP6-VP7-VP4	69,685	0	0	+	0	225
							230

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Group	Antigen	Serum Titre	Wa	Clinical Score			PRN
				DS-1	SA-11	BRV	
12	VP6 (100)	53,625	ND	ND	ND	++	<40
13	VP6 (10)	29,960	ND	ND	ND	++	<40
14	VP7 (100)	4,110	ND	ND	ND	+	<40
15	VP7 (10)	27,610	ND	ND	ND	++	<40
16	VP4 (100)	22,550	ND	ND	ND	0	600
17	VP4 (10)	35,150	0	0	+	0	540
18	VP6 + VP4	59,080	ND	ND	ND	0	>1280
19	VP6 + VP7	145,820	ND	ND	ND	+	<40
20	VP6 + VP7 + VP4	44,620	ND	ND	ND	0	460

Table 1 (cont'd)

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Table 1 (cont'd)

Group	Antigen	Serum Titre	Wa	Clinical Score		
				DS-1	SA-11	BRV
21	VP6-VP7 plus insect cell proteins	544,447	ND	ND	ND	+
						PRN <40

Group 3 is bovine rotavirus antigen and was given at a dose of 50 µg/mouse. Groups 4-6 are partially purified viral proteins. 10 µg/mouse of these proteins were administered. Groups 7-11 and 21 are assembled particles. Approximately 10 µg of VP6 and VP7 were administered per mouse and approximately 3.2 µg of VP4 administered per mouse. Groups 12-17 are partially purified viral proteins and the µg/mouse administered is in parentheses. Groups 18-20 are mixed, crude lysates. Approximately 10 µg/mouse of the mixed lysate was administered and 90 µg/mouse of the insect cell protein (Group 21). ND = Not Done  
PRN = 50% plaque reduction neutralization titers.

30 25 20 15 10 5 .

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As can be seen, vaccination with the viral particles, VP4 alone, and mixed crude cell lysates, provided protection to challenged neonatal mice.

Thus, viral particle assemblies, vaccine  
5 compositions containing these assemblies, and methods of treating and preventing rotaviral disease in a vertebrate subject are disclosed. Although preferred embodiments of the subject invention have been described in some detail,  
10 it is understood that obvious variations can be made without departing from the spirit and the scope of the intention as defined by the appended claims.



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Claims

1. A viral particle assembly capable of eliciting an immunological response in a vertebrate  
5 subject, said viral particle assembly comprising:  
    (a) an inner capsid protein substantially homologous and functionally equivalent to VP6; and  
    (b) one or more outer capsid proteins selected from the group consisting of (i) a protein substantially  
10 homologous and functionally equivalent to VP4, or a functional fragment thereof, and (ii) a protein substantially homologous and functionally equivalent to VP7.
- 15 2. The viral particle assembly of claim 1 wherein (b) comprises a protein substantially homologous and functionally equivalent to VP4, or a functional fragment thereof.
- 20 3. The viral particle assembly of claim 1 wherein (b) comprises a protein substantially homologous and functionally equivalent to VP7, or a functional fragment thereof.
- 25 4. The viral particle assembly of claim 1 wherein (b) comprises a protein substantially homologous and functionally equivalent to VP4, or a functional fragment thereof, and a protein substantially homologous and functionally equivalent to VP7, or a functional  
30 fragment thereof.
5. The viral particle assembly of claim 1 wherein (a) comprises VP6 and (b) comprises VP4.

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6. The viral particle assembly of claim 1 wherein (a) comprises VP6 and (b) comprises VP7.

7. The viral particle assembly of claim 1 wherein (a) comprises VP6 and (b) comprises VP4 and VP7.

8. The viral particle assembly of claim 1 wherein said (a) and (b) proteins are recombinantly produced.

9. A viral particle assembly capable of eliciting an immunological response in a vertebrate subject, said viral particle assembly comprising VP6 assembled with VP4 and VP7.

10. A vaccine composition comprising a pharmaceutically acceptable vehicle and the viral particle assembly of claim 1.

11. A vaccine composition comprising a pharmaceutically acceptable vehicle and the viral particle assembly of claim 9.

12. The vaccine composition of claim 10 further comprising one or more unassembled proteins selected from the group consisting of VP6, VP4 and VP7.

13. The vaccine composition of claim 11 further comprising one or more unassembled proteins selected from the group consisting of VP6, VP4 and VP7.

14. The vaccine composition of claim 12 wherein said unassembled protein is provided in a cell lysate.

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15. The vaccine composition of claim 13 wherein said unassembled protein is provided in a cell lysate.

5

16. The vaccine composition of claim 10 further comprising an adjuvant.

17. The vaccine composition of claim 11 further comprising an adjuvant.

10

18. A method of treating or preventing rotaviral disease in a vertebrate subject comprising administering to said subject a therapeutically effective amount of a vaccine composition according to claim 10.

15

19. A method of treating or preventing rotaviral disease in a vertebrate subject comprising administering to said subject a therapeutically effective amount of a vaccine composition according to claim 11.

20

20. A method of treating or preventing rotaviral disease in a vertebrate subject comprising administering to said subject a therapeutically effective amount of a vaccine composition according to claim 12.

25

21. A method of treating or preventing rotaviral disease in a vertebrate subject comprising administering to said subject a therapeutically effective amount of a vaccine composition according to claim 13.

30

Sequence Range: 1 to 1356

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10	20	30	40
GG CTT TTA AAC GAA GTC TTC AAC ATG GAT GTC CTG TAC TCC TTG			*
CC GAA AAT TTG CTT CAG AAG TTG TAC CTA CAG GAC ATG AGG AAC			
		Met Asp Val Leu Tyr Ser Leu>	
50	60	70	80
TCA AAA ACT CTT AAA GAT GCT AGA GAC AAA ATT GTC GAA GGC ACA		*	
AGT TTT TGA GAA TTT CTA CGA TCT CTG TTT TAA CAG CTT CCG TGT			
Ser Lys Thr Leu Lys Asp Ala Arg Asp Lys Ile Val Glu Gly Thr>			
90	100	110	120
* TTA TAC TCC AAT GTA AGT GAT GAT CTA ATT CAA CAA TTT AAT CAA ATG			130
AAT ATG AGG TTA CAT TCA CTA GAT TAA GTT GTT AAA TTA GTT TAC			*
Leu Tyr Ser Asn Val Ser Asp Leu Ile Gln Gln Phe Asn Gln Met>			
140	150	160	170
* ATA ATT ACT ATG AAT GGA AAT GAG TTC CAA ACT GGA GGA ATT GGT		*	
TAT TAA TGA TAC TTA CCT TTA CTC AAG GTT TGA CCT CCT TAA CCA			
Ile Ile Thr Met Asn Gly Asn Glu Phe Gln Thr Gly Ile Gly>			
180	190	200	210
* AAT CTA CCG ATT AGA AAT TGG AAT TTT GAT TTT GGA TTA CTT GGA		*	220
TTA GAT GGC TAA TCT TTA ACC TTA AAA CTA AAA CCT AAT GAA CCT			*
Asn Leu Pro Ile Arg Asn Trp Asn Phe Asp Phe Gly Leu Leu Gly>			

Figure 1 (Sheet 1 of 6)

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230 *      240      250      260      310
ACA ACT CTA CTA AAT TTA GAT GCT AAC TAC GTC GAA ACG GCC CGC
TGT TGA GAT GAT TTA AAT CTA CGA TTG ATG CAG CTT TGC CGG GCG
Thr Thr Leu Leu Asn Leu Asp Ala Asn Tyr Val Glu Thr Ala Arg>

270 *      280      290      300      350
AAT ACA AAT GAT TAT TTT GTA GAT TTT GTA AAT AAT GTA TGT ATG
TTA TGT TTA CTA ATA AAA CAT CTA AAA CAT CTA TTA CAT ACA TAC
Asn Thr Asn Asp Tyr Phe Val Asp Phe Val Asp Asn Val Cys Met>

320 *      330      340      350
GAC GAA ATG GTT AGA GAA TCA CAA AGA AAT GGA ATT GCA CCA CAA
CTG CTT TAC CAA TCT CTT AGT GTT TCT TTA CCT TAA CGT GGT GTT
Asp Glu Met Val Arg Glu Ser Gln Arg Asn Gly Ile Ala Pro Gln>

360 *      370      380      390      400
TCA GAT TCA CTT ATA AAG TTA TCA GGC ATT AAA TTT AAA AGA ATA
AGT CTA AGT GAA TAT TTC AAT AGT CCG TAA TTT AAA TTT TCT TAT
Ser Asp Ser Leu Ile Lys Leu Ser Gly Ile Lys Phe Lys Arg Ile>

410 *      420      430      440
AAT TTT GAC AAT TCA TCA GAA TAC ATA GAG AAC TGG AAT TTG CCA
TTA AAA CTG TTA AGT AGT CTT ATG TAT CTC TTG ACC TTA AAC GGT
Asn Phe Asp Asn Ser Ser Glu Tyr Ile Glu Asn Trp Asn Leu Pro>

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450 *      460 *      470 *      480 *      490 *
    AAT AGA AGA CAA AGA ACG GGT TTT ACA TTT CAT AAA CCA AAC ATT
    TTA TCT TCT GTT TCT TGC CCA AAA TGT AAA GTA TTT GGT TTG TAA
    Asn Arg Arg Gln Arg Thr Gly Phe Thr Phe His Lys Pro Asn Ile>

    500 *      510 *      520 *      530 *
    TTC CCT TAT TCA GCT TCA TTC ACG TTG AAC AGA TCA CAA CCT TCT
    AAG GGA ATA AGT CGA AGT AAG TGC AAC TTG TCT AGT GGT GGA AGA
    Phe Pro Tyr Ser Ala Ser Phe Thr Leu Asn Arg Ser Gln Pro Ser>

540 *      550 *      560 *      570 *      580 *
    CAT GAT AAC TTG ATG GGT ACG ATG TGG CTC AAT GCG GGA TCA GAA
    GTA CTA TTG AAC TAC CCA TGC TAC ACC GAG TTA CGC CCT AGT CTT
    His Asp Asn Leu Met Gly Thr Met Trp Leu Asn Ala Gly Ser Glu>

    590 *      600 *      610 *      620 *
    ATT CAG GTC GCT GGA TTC GAC TAC TCA TGT GCA ATA AAC GCG CCA
    TAA GTC CAG CGA CCT AAG CTG ATG ACG ACA CGT TAT TTG CGC GGT
    Ile Gln Val Ala Gly Phe Asp Tyr Ser Cys Ala Ile Asn Ala Pro>

630 *      640 *      650 *      660 *      670 *
    GCT AAT ACG CAA CAA TTT GAG CAT ATT GTA CAG CTT CGA AGG GTG
    CGA TTA TGC GTT GTT AAA CTC GTA TAA CAT GTC GAA GCT TCC CAC
    Ala Asn Thr Gln Gln Phe Glu His Ile Val Gln Leu Arg Arg Val>

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Figure 1 (Sheet 3 of 6)

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680	*	690	*	700	*	710	*							
TTG	ACT	ACA	GCT	ACA	ATA	ACT	CTT	TTA	CCA	GAT	GCA	AGA	AGA	TTT
AAC	TGA	TGT	CGA	TGT	TAT	TGA	GAA	AAT	GGT	CTA	CGT	CTT	TCT	AAA
Leu	Thr	Thr	Ala	Thr	Ile	Thr	Leu	Leu	Pro	Asp	Ala	Glu	Arg	Phe>
720	*	730	*	740	*	750	*	760	*					
AGT	TTT	CCA	AGA	GTG	ATT	ACT	TCA	GCT	GAC	GGA	GCG	ACT	ACA	TGG
TCA	AAA	GGT	TCT	CAC	TAA	TGA	AGT	CGA	CTG	CCT	CGC	TGA	TGT	ACC
Ser	Phe	Pro	Arg	Val	Ile	Thr	Ser	Ala	Asp	Gly	Ala	Thr	Thr	Trp>
770	*	780	*	790	*	800	*							
TAC	TTC	AAT	CCA	GTG	ATT	CTT	AGA	CCA	AAT	AAC	GTT	GAA	ATA	GAG
ATG	AAG	TTA	GGT	CAC	TAA	GAA	TCT	GGT	TTA	TTG	CAA	CTT	TAT	CTC
Tyr	Phe	Asn	Pro	Val	Ile	Leu	Arg	Pro	Asn	Asn	Val	Glu	Ile	Glu>
810	*	820	*	830	*	840	*	850	*					
TTT	CTA	CTA	AAC	GGG	CAG	ATA	ATA	AAT	ACT	TAC	CAA	GCA	AGA	TTT
AAA	GAT	GAT	TTG	CCC	GTC	TAT	TAT	TTA	TGA	ATG	GTT	CGT	TCT	AAA
Phe	Leu	Leu	Asn	Gly	Gln	Ile	Ile	Asn	Thr	Tyr	Gln	Ala	Arg	Phe>
860	*	870	*	880	*	890	*							
GGA	ACC	ATC	ATA	GCT	AGA	AAT	TTT	GAT	ACA	ATT	AGA	TTG	TCA	TTT
CCT	TGG	TAG	TAT	CGA	TCT	TTA	AAA	CTA	TGT	TAA	TCT	AAC	AGT	AAA
Gly	Thr	Ile	Ile	Ala	Arg	Asn	Phe	Asp	Thr	Ile	Arg	Leu	Ser	Phe>

Figure 1 (Sheet 4 of 6)

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900	*	910	*	920	*	930	*	940	*
CAG	TTG	ATG	AGA	CCA	CCA	AAT	ATG	ACA	CCA
GTC	AAC	TAC	TCT	GGT	TTA	TAC	TGT	CGC	GTA
Gln	Leu	Met	Arg	Pro	Pro	Asn	Met	Thr	Pro
									Ala
									Ala
									Leu>
950	*	960	*	970	*	980	*		
TTT	CCA	AAT	GCG	CAG	CCA	TTT	GAA	CAT	CAC
AAA	GGT	TTA	CGC	GTC	GGT	AAA	CTT	GTA	GTG
Phe	Pro	Asn	Ala	Gln	Pro	Phe	Glu	His	Ala
									Thr
									Val
									Gly
									Leu>
990	*	1000	*	1010	*	1020	*	1030	*
ACG	CTT	AGA	ATT	GAA	TCT	GCA	GTT	TGT	GAA
TGC	GAA	TCT	TAA	CTT	AGA	CGT	CAA	ACA	CTT
Thr	Leu	Arg	Ile	Glu	Ser	Ala	Val	Cys	Glu
									Ser
									Val
									Leu
									Ala
									Asp>
1040	*	1050	*	1060	*	1070	*		
GCA	AGC	GAA	ACA	ATG	CTA	GCA	AAT	GTG	ACA
CGT	TCG	CTT	TGT	TAC	GAT	CGT	TTA	CAC	TGT
Ala	Ser	Glu	Thr	Met	Leu	Ala	Asn	Val	Thr
									Ser
									Val
									Arg
									Gln
									Glu>
1080	*	1090	*	1100	*	1110	*	1120	*
TAC	GCG	ATA	CCA	GTT	GGA	CCA	GTT	TTT	CCA
ATG	CGC	TAT	GGT	CAA	CCT	GGT	CAA	AAA	GGT
Tyr	Ala	Ile	Pro	Val	Gly	Pro	Val	Phe	Pro
									Pro
									Gly
									Met
									Asn
									Trp>

Figure 1 (Sheet 5 of 6)

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1130	1140	1150	1160
ACT GAT TTG ATC ACT AAC TAT TCA CCA TCT AGA GAG GAT AAC TTG			
TGA CTA AAC TAG TGA TTG ATA AGT GGT AGA TCT CTC CTA TTG AAC			
Thr Asp Leu Ile Thr Asn Tyr Ser Pro Ser Arg Glu Asp Asn Leu>			
1170	1180	1190	1200
CAG CGT GTA TTT ACA GTG GCT TCC ATT AGA AGC ATG CTT GTC AAA			
GTC GCA CAT AAA TGT CAC CGA AGG TAA TCT TCG TAC GAA CAG TTT			
Gln Arg Val Phe Thr Val Ala Ser Ile Arg Ser Met Leu Val Lys>			
1220	1230	1240	1250
TGA GGA CCA AGC TAA CCA CTT GGT ATC CGA CTT TGG TGA GTA TGT			
ACT CCT GGT TCG TCG ATT GGT GAA CCA TAG GCT GAA ACC ACT CAT ACA			
1260	1270	1280	1290
AGC TAC GTC AAG CTG TTT GAA CTC TGT AAG TAA GGA TGC GTC TAC			
TCG ATG CAG TTC GAC AAA CTT GAG ACA TTC ATT CCT ACG CAG ATG			
1310	1320	1330	1340
GTA TTC GCT ACA CAG AGT AAT CAC TCA GAT GGC GTA GTG AGA GGA			
CAT AAG CGA TGT GTC TCA TTA GTG AGT CTA CCG CAT CAC TCT CCT			
1350			
TGT GAC C			
ACA CTG G			

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Sequence Range: 1 to 451

	10	20	30	40	50
	*	*	*	*	*
Translatio	LLNEV FNMDV	LYSLS KTLKD ARDKI	VEGTL YSNVS	DLIQQ FNQMI	ITMNG
ROBMCP	15	30	45	60	75
[ 2164 ]	LLNEV FNMDV	LYSLS KTLKD ARDKI	VEGTL YSNVS	DLIQQ FNQMI	ITMNG>
	^	^	^	^	^
RO1HVP6	15	30	45	60	75
[ 2061 ]	LLNEV FNMDV	LYSLS KTLKD ARDKI	VEGTL YSNVS	DLIQQ FNQMI	ITMNG>
	^	^	^	^	^
RO1VVP6H2	15	30	45	60	75
[ 1997 ]	LLNEV FNMDV	LYSLS KTLKD ARDKI	VEGTL YSNVS	DLIQQ FNQMV	ITMNG>
	^	^	^	^	^
RO1VVP6FI	15	30	45	60	75
[ 1974 ]	LLNEV FNMeV	LYSIS KTLKD ARDKI	VEGTL YSNVS	DLIQQ FNQII	VTMNG>
	^	^	^	^	^
RO1PVP6	15	30	45	60	75
[ 1949 ]	LLNEV FNMeV	LYSLS KTLKD ARDKI	VEGTL YSNVS	DLIQQ FNQMI	VTMNG>
	^	^	^	^	^
RO2SEG6	15	30	45	60	75
[ 1939 ]	L*NEV FdMeV	LYSLS KTLKD ARDKI	VEGTL YSNVS	DLIQQ FNQMI	VTMNG>
	^	^	^	^	^
PRVVP6	25	40	55	70	85
[ 904 ]	FtMDV LfSia	KTVSD lkkKv	VvGTi YtNve	DLIQQ tneli	rTING>
	^	^	^	^	^

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Translatio	60	70	80	90	100					
	* GIGN	* LPIRN	* WNFD	* GLLGT	* TLLNL	* DANYV	* ETARN	* TNDYF	* VDFVD	
ROBMCP	165	180	195	210	225	240	255	270	285	300
[ [ 2164 ]	NEFQT	GGIGN	LPIRN	WNFD	GLLGT	TLLNL	DANYV	ETARN	TiDYF	VDFVD>
	NEFQT	GGIGN	LPIRN	WNFD	GLLGT	TLLNL	DANYV	ETARN	TiDYF	VDFVD>
	NEFQT	GGIGN	LPIRN	WNFD	GLLGT	TLLNL	DANYV	ETARN	TiDYF	VDFVD>
RO1HVP6	165	180	195	210	225	240	255	270	285	300
[ [ 2061 ]	NEFQT	GGIGN	LPIRN	WNFD	GLLGT	TLLNL	DANYV	ETARN	TiDYF	VDFVD>
	NEFQT	GGIGN	LPIRN	WNFD	GLLGT	TLLNL	DANYV	ETARN	TiDYF	VDFVD>
RO1VVP6H2	165	180	195	210	225	240	255	270	285	300
[ [ 1997 ]	NEFQT	GGIGN	LPIRN	WNFD	GLLGT	TLLNL	DANYV	ETARN	TiDYF	VDFVD>
	NEFQT	GGIGN	LPIRN	WNFD	GLLGT	TLLNL	DANYV	ETARN	TiDYF	VDFVD>
RO1VVP6FI	165	180	195	210	225	240	255	270	285	300
[ [ 1974 ]	NEFQT	GGIGN	LPIRN	WNFD	GLLGT	TLLNL	DANYV	ETARN	TiDYF	VDFVD>
	NEFQT	GGIGN	LPIRN	WNFD	GLLGT	TLLNL	DANYV	ETARN	TiDYF	VDFVD>
RO1PVP6	165	180	195	210	225	240	255	270	285	300
[ [ 1949 ]	NdFQT	GGIGN	LPVRN	WtFDF	GLLGT	TLLNL	DANYV	ETARN	TieyF	iDFiD>
	NdFQT	GGIGN	LPVRN	WtFDF	GLLGT	TLLNL	DANYV	ETARN	TieyF	iDFiD>
RO2SEG6	165	180	195	210	225	240	255	270	285	300
[ [ 1939 ]	NdFQT	GGIGN	LPVRN	WtFDF	GLLGT	TLLNL	DANYV	ETARN	TieyF	iDFiD>
	NdFQT	GGIGN	LPVRN	WtFDF	GLLGT	TLLNL	DANYV	ETARN	TieyF	iDFiD>
PRVVP6	160	175	190	205	220	235	250	265	280	295
[ [ 904 ]	NtFht	GGIGN	qpqke	WNFql	pqLGT	TLLNL	DdNYV	qatRS	viDYl	asFie>
	NtFht	GGIGN	qpqke	WNFql	pqLGT	TLLNL	DdNYV	qatRS	viDYl	asFie>

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			110	*	120	*	130	*	140	*	150	*
Translatio	NVCMD	EMVRE	SQRNG	IAPQS	DSLIK	LSGIK	FKRIN	FDNSS	EYIEN	WNLPN		
	315	330	345	360	375	390	405	420	435	450		
ROBMCP [ [ 2164 ]	NVCMD	EMVRE	SQRNG	IAPQS	DSLIK	LSGIK	FKRIN	FDNSS	EYIEN	WNLPN>		
	315	330	345	360	375	390	405	420	435	450		
RO1HVP6 [ [ 2061 ]	NVCMD	EMVRE	SQRNG	IAPQS	DSLrK	LSGIK	FKRIN	FDNSS	EYIEN	WNLqN>		
	315	330	345	360	375	390	405	420	435	450		
RO1VVP6H2 [ [ 1997 ]	NVCMD	EMVRE	SQRNG	IAPQS	DSLrK	LSGIK	FKRIN	FDNSS	EYIEN	WNLqN>		
	315	330	345	360	375	390	405	420	435	450		
RO1VVP6FI [ [ 1974 ]	NVCMD	EMtRE	SQRNG	IAPQS	DalrK	LSGIK	FKRIN	FDNSS	EYIEN	WNLqN>		
	315	330	345	360	375	390	405	420	435	450		
RO1PVP6 [ [ 1949 ]	NVCMD	EMaRE	SQRNG	IAPQS	eafRK	LaGIK	FKRIN	FDNSS	EYIEN	WNLqN>		
	315	330	345	360	375	390	405	420	435	450		
RO2SEG6 [ [ 1939 ]	NVCMD	EMaRE	SQRNG	VAPQS	eaLrK	LaGIK	FKRIN	FDNSS	EYIEN	WNLqN>		
	315	330	345	360	375	390	405	420	435	450		
PRVVP6 [ [ 904 ]	aVCdD	EiVRE	asRNG	mQPQS	pTLia	LassK	FkTIN	FnNSS	qsIKN	ws-aq>		
	310	325	340	355	370	385	400	415	430	445		

Figure 2 (Sheet 3 of 10)

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[illegible]

Figure 2 (Sheet 5 of 10)

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[illegible]

Figure 2 (Sheet 6 of 10)

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RO1HVP6  
[ 2061 ]

RO1VVP6H2  
[ 1997 ]

RO1VVP6FI  
[ 1974 ]

ROI PVP6  
[ 1949 ]

RO2SEG6  
[ 1939 ]

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PRVVP6      760  775  790  805  820  835  850  865  880
[  904 ]    qTTW1 yNPdq Lmngt ftIEF ynNGQ lvdmv r-nmG vvtvR tFDsy Ritid>
          ^^^  ^^^^v ^  ^^^^ v^^^ v  ^  ^  ^  ^  ^  ^  ^  ^

Translation  310      *      320      *      330      *      340      *      350
          LMRPP NMTPA VAALF PNAQP FEHHA TVGLT LRIES AVCES VLADA SETML
          915  930  945  960  975  990  1005  1020  1035  1050
ROBMCP      LMRPP NMTPA VAALF PNAQP FEHHA TVGLT LRIES AVCES VLADA SETML>
[ 2164 ]    ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^

RO1HVP6      915  930  945  960  975  990  1005  1020  1035  1050
[ 2061 ]    LMRPP NMTPt VAALF PNAQP FEHHA TVGLT LRIES AVCES VLADA SETML>
          ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^

pp
|
RO1VVP6H2    930  945  960  975  990  1005  1020  1035  1050
[ 1997 ]    LMRPq NMTPA VAALF PNApP FEHHA TVGLT LRIES AiCES VLADA SETML>
          ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^

RO1VVP6FI    915  930  945  960  975  990  1005  1020  1035  1050
[ 1974 ]    LMRPP NMTPA VnALF PqAQP FqHHA TVGLT LRIbS AVCES VLADs nETML>
          ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^

RO1PVP6      915  930  945  960  975  990  1005  1020  1035  1050
[ 1949 ]    LMRPP NMTPA VdALF PqAQP FqHHA TVGLT LRIES AVCES VLADA nETIL>
          ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^  ^^^^

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Accession	1915	1930	1945	1960	1975	1990	2005	2020	2035	2050
RO2SEG6 [ 1939 ]	LMRPP ^ ^ ^ ^	NMTPA ^ ^ ^ ^	VnALF ^ ^ ^ ^	PqAQp ^ ^ ^ ^	FqHHA ^ ^ ^ ^	TVGLT ^ ^ ^ ^	LRIES ^ ^ ^ ^	AVCES ^ ^ ^ ^	VLADA ^ ^ ^ ^	nETIL> ^ ^ ^ ^
PRVVP6 [[ 904 ]	895 ^ ^ ^ ^	910 aMTqy ^ ^ v ^	925 VqrLF ^ v ^ ^	940 Pqggp ^ ^ ^ ^	955 ypyqa ^ ^ ^ ^	970 aymLT v v ^ ^	985 Lsild ^ ^ v ^	1000 Attes ^ v ^ ^	1015 VLCds ^ ^ v ^	1030 hsvdv> v ^ ^ ^
Translatio	ANvTS	VRQEy	AIPVG	PVFPP	GMNWT	DLITN	YSPSR	EDNLQ	RVFTV	ASIRS
ROBMCP [ 2164 ]	1065 ANvTS	1080 VRQEy	1095 AIPVG	1110 PVFPP	1125 GMNWT	1140 DLITN	1155 YSPSR	1170 EDNLQ	1185 RVFTV	1200 ASIRS>
RO1HVP6 [ 2061 ]	1065 ANvTS	1080 VRQEy	1095 AIPVG	1110 PVFPP	1125 GMNWT	1140 DLITN	1155 YSPSR	1170 EDNLQ	1185 RVFTV	1200 ASIRS>
RO1VVP6H2 [ 1997 ]	1065 ANvTS	1080 VRQEy	1095 AvpVG	1110 PVFPP	1125 GMNWT	1140 DLITN	1155 YSPSR	1170 EDNLQ	1185 RVFTV	1200 ASIRS>
RO1VVP6FI [ 1974 ]	1065 ANvTa	1080 VRQEy	1095 AvpVG	1110 PVFPP	1125 GMNWT	1140 eLITN	1155 YSPSR	1170 EDNLQ	1185 RVFTV	1200 ASIRS>
RO1PVP6 [ 1949 ]	1065 ANvTa	1080 VRQEy	1095 AIPVG	1110 PVFPP	1125 GMNWT	1140 eLITN	1155 YSPSR	1170 EDNLQ	1185 RVFTV	1200 ASIRS>

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[illegible]

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RO2SEG6      1215 1230 1245 1260 1275 1290 1305 1320 1335 1350
[ 1939 ]     MLIK* Gpd*a sGIqs *lacs yIKSF rLFK* Ghdfm FAT*S Nclnd VVRGC>
            ^^^^V ^^ V V^^ V VV ^^ ^^V^^ ^V^V ^V^V ^V^V ^V^V ^V^V
            195
PRVVP6      [ 904 ]     Mvm>
            ^^^

Translatio  D
ROBMCP      [ 2164 ]     D> ^
RO2SEG6      [ 1939 ]     D> ^
    
```

Figure 2 (Sheet 10 of 10)

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Sequence Range: 1 to 776

C486G4p	MASLI YRQLL TNSYT VELSD EIQEI GSTKT QNVTV NPGPF AQTNY ASVNW	10	20	30	40	50
		*	*	*	*	*
SA11G4p	5 10 15 20 25 30 35 40 45 50					
[ 3506 ]	MAaLI YRQLL TNSYT VELSD EIQEI GSTKT QNVTV NPGPF AQTNY ApVNW>					
	^^ ^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^					
RRVVP4p	5 10 15 20 25 30 35 40 45 50					
[ 3198 ]	MASLI YRQLL TNSYT VdLSD EIQEI GSTKT QNVTi N1GPF AQTgY ApVNW>					
	^^ ^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^					
DS-1p	5 10 15 20 25 30 35 40 45 50					
[ 2870 ]	MASLI YRQLL TNSYS VdLhD E1eqI GSeKT QsVTV NPGPF AQTry ApVNW>					
	^^ ^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^					
M37p	5 10 15 20 25 30 35 40 45 50					
[ 2844 ]	MASLI YRQLL TNSYS VELSD EIntI GSeKT QNVTi NPGPF AQTNY ApVv1>					
	^^ ^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^					
KUp	5 10 15 20 25 30 35 40 45 50					
[ 2837 ]	MASLI YRQLL TNSYS VdLhD E1eqI GSeKT QNVTV NPGPF AQTry ApVNW>					
	^^ ^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^					
ST3p	5 10 15 20 25 30 35 40 45 50					
[ 2836 ]	MASLI YRQLL TNSYT VELSD EIntI GSeKS QNiTi NPGPF AQTNY ApVv1>					
	^^ ^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^					
K8p	5 10 15 20 25 30 35 40 45 50					
[ 2735 ]	MASLI YRQLL sNSYV tniSD EvneI GtKKT tNVTV NPGPF AQTgY ApVdW>					
	^^ ^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^ ^^^^					

Figure 3 (Sheet 1 of 17)

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C48G4p	GPGET	NDSTT	VEPVL	DGPYQ	PTTFN	PPVSY	WMLLA	PTNAG	VVDQG	TNNTN
SA11G4p	GPGET	NDSTT	VEPVL	DGPYQ	PTTFN	PPVSY	WMLLA	PTNAG	VVveG	TNNTN>
[ 3506 ]	^^^^	^^^^	^^^^	^^^^	^^^^	^^^^	^^^^	^^^^	^^	^^^^
RRVVP4p	GPGET	NDSTT	VEPVL	DGPYQ	PTsFN	PPVdy	WMLLA	PTaAG	VVveG	TNNTd>
[ 3198 ]	^^^^	^^^^	^^^^	^^^^	^^	^^	^^^^	^^	^^	^^^^
DS-1p	ChGEi	NDSTT	VEPVL	DGPYQ	PTTFk	PpTdy	Wllis	sntng	VVyes	TNNnd>
[ 2870 ]	^	^^^^	^^^^	^^^^	^^^^	^^	^^	^^	^^	^^
M37p	eswEv	NDSTT	iEPVL	DGPYQ	P-TFk	PpTdy	Willn	PTdqq	VVleg	TNkTd>
[ 2844 ]	^	^^^^	^^^^	^^^^	^^	^^	^^^^	^^	^^	^^
KUP	ChGEi	NDSTT	VEPiL	DGPYQ	PTTFk	PlTdy	WlIn	sntng	VVyes	TNNSd>
[ 2837 ]	^	^^^^	^^^^	^^^^	^^^^	^^	^^^^	^^	^^	^^
ST3p	eswEv	NDSTT	iEPVL	DGPYQ	P-TFk	PpTdy	Willn	PTNqq	VVleg	TNkTd>
[ 2836 ]	^	^^^^	^^^^	^^^^	^^	^^	^^^^	^^	^^	^^

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K8p      55      60      65      70      75      80      85      90      95      100
[ 2735 ]  ChGE1  pDSt1  VqPtL  DGPYQ  PTLn1  PvtDy  WMLiA  PtReG  kVaeG  TNtTd>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

C486G4p   110      *      120      *      130      *      140      *      150
          RWLAT  ILIKP  NVQQV  ERTYT  LFGQQ  VQVTV  SNDSQ  TKWKF  VDLSK  QTQDG
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

SA11G4p   105      110      115      120      125      130      135      140      145      150
[ 3506 ]  RWLAT  ILIEP  NVQQV  ERTYT  LFGQQ  VQVTV  SNDSQ  TKWKF  VDLSK  QTQDG>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

RRVVP4p   105      110      115      120      125      130      135      140      145      150
[ 3198 ]  RWLAT  ILVeP  NVtse  tRsYT  LFGtQ  eQiTi  aYaSQ  TqWKF  iDvVK  tTQnG>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

DS-1p     105      110      115      120      125      130      135      140      145
[ 2870 ]  fWtAV  IaIeP  hVsQV  nRqYT  LFGen  kQfnV  eNnSd  -KWKF  femfK  gssqG>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

M37p      105      110      115      120      125      130      135      140      145
[ 2844 ]  iWiAl  LLVeP  NVtnq  sRqYT  LFGet  kQiTV  eNntn  -KWKF  femfr  knvsa>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

KUP        105      110      115      120      125      130      135      140      145
[ 2837 ]  fWtAV  vaIeP  hViQV  dRqYT  vFGen  kQfnV  rNDSD  -KWKF  lemfr  gssqn>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

ST3p       105      110      115      120      125      130      135      140      145
[ 2836 ]  iWiAl  LLVeP  NVtnq  sRqYT  LFGet  kQiTV  eNntn  -KWKF  femfr  ssvss>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

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K8p		105	110	115	120	125	130	135	140	145	150
[ [ 2735 ]	RWfAc vLveP NVQnt qRqYv LdGQn VQLhv SNDSS TSWKF ilfik lTyg>	^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^									
C486G4p		160	*	170	*	180		190	*	200	*
	NYSQH GPLLS TPKLY GVMKH GKkiY TYNGE TPNAT TGYYS TTnFD TVNMT										
SA11G4p		155	160	165	170	175	180	185	190	195	200
[ [ 3506 ]	NYSQH GSLLS TPKLY GVMKH GKkiY TYNGE TPNAN TGYYS TTnFD TVNMT>	^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^									
RRRVVP4p		155	160	165	170	175	180	185	190	195	200
[ [ 3198 ]	sVSQy GPLqs TPKLy aVmkH nGkiY TYNGE TPnVT TkYYS TTnyD sVNMT>	^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^									
DS-1p		150	155	160	165	170	175	180	185	190	195
[ [ 2870 ]	NfSnr rtLtS snrLv GmlKy GGrvw TfHgE TPPrAT Tdssn TaDln nisi>	^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^									
M37p		150	155	160	165	170	175	180	185	190	195
[ [ 2844 ]	efghk rtLtS dtKLd GflKH ynsvw TfHgE TPPhAT TDySS TSnlS eVeTv>	^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^									
KUp		150	155	160	165	170	175	180	185	190	195
[ [ 2837 ]	efynr rtLtS dtKLv GilKy GGriw TfHgE TPPrAT Tdssn TanLn disii>	^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^									
ST3p		150	155	160	165	170	175	180	185	190	195
[ [ 2836 ]	efghk rtLtS dtKLd GflKH ynsvw sfHgE TPPhAT TDySS TSnlS eVeTv>	^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^									

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	155	160	165	170	175	180	185	190	195	200
K8p	155	160	165	170	175	180	185	190	195	200
[ [ 2735 ]	tytQy	stLst	phKLc	awMKr	dnrvY	wYqGa	TPNAs	esYyI	TiNnD	nsNvs>
	^	^	^^	^^	^^	^	^^	^^	^	^^
		210	*	220	*	230	*	240	*	250
C486G4p	AYCDF	YIIPL	AQEAk	CTEYI	NNGLP	PIQNT	RNIVP	VSIVS	RNIVY	TRAQP
SA11G4p	205	210	215	220	225	230	235	240	245	250
[ [ 3506 ]	AYCDF	YIIPL	AQEAk	CTEYI	NNGLP	PIQNT	RNIVP	VSIVS	RNIVY	TRAQP>
	^^^	^^^	^^^	^^^	^^^	^^^	^^^	^^^	^^^	^^^
RRVVP4p	205	210	215	220	225	230	235	240	245	250
[ [ 3198 ]	AfCDF	YIIPr	eeEst	CTEYI	NNGLP	PIQNT	RNIVP	lalsa	RNIis	hRAQa>
	^^^	^^^	^^	^^^	^^^	^^^	^^^	^^	^^^	^^^
DS-1p	200	205	210	215	220	225	230	235	240	245
[ [ 2870 ]	ihseF	YIIPr	sQEsK	CnEYI	NNGLP	PIQNT	RNVVP	lSlSS	RsIqY	rRAQV>
	^^	^^^	^^	^^	^^^	^^^	^^^	^^	^^	^^
M37p	200	205	210	215	220	225	230	235	240	245
[ [ 2844 ]	ihveF	YIIPr	sQEsK	CvEYI	NtGLP	PmQNT	RNIVP	ValSS	RsvtY	qRAQV>
	^^	^^^	^^	^^	^^	^^^	^^^	^^	^^	^^
KUP	200	205	210	215	220	225	230	235	240	245
[ [ 2837 ]	ihseF	YIIPr	sQEsK	CnEYI	NNGLP	PIQNT	RNVVP	lSlSS	RsIqY	kRAQV>
	^^	^^^	^^	^^	^^^	^^^	^^^	^^	^^	^^
ST3p	200	205	210	215	220	225	230	235	240	245
[ [ 2836 ]	ihveF	YIIsr	sQEsK	CvEYI	NtGLP	PmQNT	RNIVP	ValSS	RsvtY	qRAQV>
	^^	^^^	^^	^^	^^^	^^^	^^^	^^	^^	^^

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K8p      205 210 215 220 225 230 235 240 245 250
[ 2735 ] sdaef YLIPq sQtAm CTqYI NNGLP PIQNT RNIVP VnITS RqIKd vRAQm>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          260 265 270 275 280 285 290 295 300
          *  *  *  *  *  *  *  *  *
C486G4p  NQDIV VSKTS LWKEM QYNRD IVIRF KFANS IIKSG GLGYK WSEVS FKPAN
          255 260 265 270 275 280 285 290 295 300
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
SA11G4p  NQDIV VSKTS LWKEM QYNRD IVIRF KFANS IIKSG GLGYK WSEVS FKPAf>
[ 3506 ] ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          255 260 265 270 275 280 285 290 295 300
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
RRVP4p   NeDIV VSKTS LWKEM QYNRD ItIRF KFASS IVKSG GLGYK WSEiS FKPAN>
[ 3198 ] ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          250 255 260 265 270 275 280 285 290 295
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
DS-1p    NeDit iSKTS LWKEM QYNRD IiIRF KFgNS ViKlG GLGYK WSEiS yKaAN>
[ 2870 ] ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          250 255 260 265 270 275 280 285 290 295
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
M37p     NeDii iSKTS LWKEM QcNRD IiIRF KFnNS IvKlG GLGYK WSEiS FKaAN>
[ 2844 ] ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          250 255 260 265 270 275 280 285 290 295
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
KUP       NeDit iSKTS LWKEM QcNRD IiIRF KFgNS IvKlG GLGYK WSEiS yKaAN>
[ 2837 ] ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          250 255 260 265 270 275 280 285 290 295
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
ST3p     NeDii iSKTS LWKEM QYNRD IiIRF KFnNS IIKlG GLGYK WSEiS FKaAN>
[ 2836 ] ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          250 255 260 265 270 275 280 285 290 295
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

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K8p      255  260  265  270  275  280  285  290  295  300
[ 2735 ]  NeDIV iSKTS LWKEM QYNRD IiIRF KFANS IIKSG GLGYK WSEiS FKpMn>
          ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^
          310  *      320  *      330  *      340  *      350  *
C486G4p  YQYTY TRDGE EVTAH TTCSV NGIND FNYNG GSLPT DFVIS KYEVI KENSF
          305  310  315  320  325  330  335  340  345  350
SA11G4p  YQYTY TRDGE EVTAH TTCSV NGvND FNYNG GSLPT DFVIS KYEVI KENSF>
[ 3506 ]  ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^
          305  310  315  320  325  330  335  340  345  350
RRVVP4p  305  310  315  320  325  330  335  340  345  350
[ 3198 ]  YQYTY TRDGE qVTAH TTCSV NGmND FNfNG GSLPT DFfIS rYEVI KENSy>
          ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^
          300  305  310  315  320  325  330  335  340  345
DS-1p    300  305  310  315  320  325  330  335  340  345
[ 2870 ]  YQYsY sRDGE qVTAH TTCSV NGvNn FSYNG GSLPT DFsIS rYEVI KENSy>
          ^^^ ^  ^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^
          300  305  310  315  320  325  330  335  340  345
M37p     300  305  310  315  320  325  330  335  340  345
[ 2844 ]  YQYnY lRDGE qVTAH TTCSV NGvNn FSYNG GSLPT DFsvS rYEVI KENSy>
          ^^^ ^  ^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^
          300  305  310  315  320  325  330  335  340  345
KUp       300  305  310  315  320  325  330  335  340  345
[ 2837 ]  YQYnY lRDGE qVTAH TTCSV NGvNn FSYNG GSLPT DFsvS rYEVI KENSy>
          ^^^ ^  ^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^
          300  305  310  315  320  325  330  335  340  345
ST3p     300  305  310  315  320  325  330  335  340  345
[ 2836 ]  YQYnY lRDGE qVTAH TTCSV NGvNn FSYNG GLLPT hfsvS rYEVI KENSy>
          ^^^ ^  ^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^

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K8p      355 360 365 370 375 380 385 390 395 400
[ 2735 ] VYVDY WDDSQ AFRNM VYVRS LAAnL NdVVC TGGsY SPALP VGNhP VMsGG>
          ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
          410 *      420 *      430 *      440 *      450 *
C486G4p  AVSLH SAGVT LSTQF TDFVS LNSLR FRFRL SVEEP PFSIL RTRVS GLYGL
          405 410 415 420 425 430 435 440 445 450
SA11G4p  AVSLH SAGVT LSTQF TDFVS LNSLR FRFRL SVEEP PFSIL RTRVS GLYGL>
[ 3506 ] ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
          405 410 415 420 425 430 435 440 445 450
RRVVP4p  AVSLH SAGVT LSTQF TDFVS fNSLR FRFRL tVEEP sFSIt RTRVg GLYGL>
[ 3198 ] ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
          400 405 410 415 420 425 430 435 440 445
DS-1p    400 405 410 415 420 425 430 435 440 445
[ 2870 ] AVSLH fAGVT LSTQF TDFVS LNSLR FRFSL tvDEP sFSIL RTRti nLYGL>
          ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
          400 405 410 415 420 425 430 435 440 445
M37p     400 405 410 415 420 425 430 435 440 445
[ 2844 ] AVSLH fAGVT LSTeF TDFVS LNSLR FRFSL tVEEP PFSIL RTRVS GLYGL>
          ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
          400 405 410 415 420 425 430 435 440 445
KUp      400 405 410 415 420 425 430 435 440 445
[ 2837 ] AVSLH fAGVT LSTQF TDFVS LNSLR FRFSL tvDEP sFSIL RTRtv nLYGL>
          ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
          400 405 410 415 420 425 430 435 440 445
ST3p     400 405 410 415 420 425 430 435 440 445
[ 2836 ] AVSLH fAGVT LSTkF TDFVS LNSLR FRFSL tVEEP PFSIL RTRVS GLYGL>
          ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^

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K8p [ [ 2735 ] ]	405    410    415    420    425    430    435    440    445    450 AVeLt SAGVT LSTQY TDyVS INSLq FRFRL avSEP sFSIS RTRMS GiVGL> ^
C486G4p	460    *    470    *    480    *    490    *    500    * PAAKP NNSQE YYEIA GRFSL ISLVP SNDDY QTPii NSVTV RQDLE RQLGE
SAl1G4p [ [ 3506 ] ]	455    460    465    470    475    480    485    490    495    500 PAAKP NNSQE YYEIA GRFSL ISLVP INDDY QTPim NSVTV RQDLE RQLGE> ^
RRVVP4p [ [ 3198 ] ]	455    460    465    470    475    480    485    490    495    500 PAAyP NNgKE YYEvA GRISL ISLVP SNDDY QTPIt NSVTV RQDLE RQLGE> ^
DS-1p [ [ 2870 ] ]	450    455    460    465    470    475    480    485    490    495 PAAnP NNgnE YYEmS GRFSL ISLVq tNDDY QTPim NSVTV RQDLE RQLnd> ^
M37p [ [ 2844 ] ]	450    455    460    465    470    475    480    485    490    495 PAfnP Nsghe YYEIA GRFSf ILLVP SNDDY QTPim NSVTV RQDLE RQLgd> ^
KUP [ [ 2837 ] ]	450    455    460    465    470    475    480    485    490    495 PAAnP NNgnE YYEIc GRFSL ISLVP tNDDY QTPim NSVTV RQDLE RQLtd> ^
ST3p [ [ 2836 ] ]	450    455    460    465    470    475    480    485    490    495 PASnP Nsghe YYEIA GRFSL ISLVP SNDDY QTPim NSitV RQDLE RQLgd> ^

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[illegible]

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[illegible]

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[illegible]

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K8p
[ [ 2735 ] dlrm qdvST QvSv SRnLR LKEft TQTdt lSFDD ISAav LKTKl DKSTQ>
600 605 610 615 620 625 630 635 640 645

```

C486G4p	LNTNT	LPEIV	TEASE	KFIPN	RAYRV	IKDDE	VLEAS	TDGKY	FAYKV	ETILK
		*		*		*		*		*
	660	660	670	670	680	680	690	690	700	700

[illegible]

```

RRVVP4p
ispNT LPdIV TEASE KFIPN RAYRV InnDE vFEAg TDGrY FAYrV ETfde>
655 660 665 670 675 680 685 690 695 700
i |

```

```
DS-1p      650   655   660   665   670   675   680   685   690   695  
            igkNT LPdIV TEASE KFIPK RSYRV lKDDE VmEin TeGkf FAYKV dTlne>  
            ^    ^    ^    ^    ^    ^    ^    ^    ^    ^  
            i |
```

```
M37P      650   655   660   665   670   675   680   685   690   695  
[ [ 2844 ]    ispdt LPdim TESsk KFIPk RAYRV lKDDE VmEAd vDGkf FAYKV dtfee>  
v |
```

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KUP      650  655  660  665  670  675  680  685  690  695
[ 2837 ]  igkNT LPdIV TEASE KFIPk RSYRi LKDEE VmEin TeGKV FAYKi dTlne>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          v |

ST3p     650  655  660  665  670  675  680  685  690  695
[ 2836 ]  irpdT LPdIi TESSE KFIPk RAYRV LKDEE VmEAd vDGKf FAYKV dTfee>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          v |

K8p      650  655  660  665  670  675  680  685  690  695
[ 2735 ]  isqqT mPdIi aEsSE KFIPk RSYRi vdeDi rfEtg iDGtf YAYKV dTfne>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          i |

C486G4p  710      720      730      740      750
          *      *      *      *      *
RFHSM YKFAD LVTDS PVISA IIDFK TLKNL NDNYG ISRQQ ALNLL RSDPR

SA11G4p  705  710  715  720  725  730  735  740  745  750
[ 3506 ]  pFd-v qKFAD LVTDS PVISA IIDFK TLKNL NDNYG ISRQQ ALNLL RSDPR>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

RRVVP4p  705  710  715  720  725  730  735  740  745  750
[ 3198 ]  pFd-v qKFAD LVTDS PVISA IIDFK TLKNL NDNYG ISRQQ AfNLL RSDPR>
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^
          ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^  ^

DS-lp    705  710  715  720  725  730  735  740  745

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[ [ 2870 ]      pFd-i nKFAe LVTDS PVISA IIDFK TLKNL NDNYG ItRie AfNLi kSnPn>
    ^   ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
M37p          705 710 715 720 725 730 735 740 745
[ [ 2844 ]      pFd-v dKFvD LVTDS PVISA IIDFK TLKNL NDNYG ItRsQ ALdLi RSDPR>
    ^   ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
KUP           705 710 715 720 725 730 735 740 745
[ [ 2837 ]      pFd-v nKFAe LVtnS PVISA IIDFK TLKNL NDNYG ItRie ALNLi kSnPn>
    ^   ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
ST3p          705 710 715 720 725 730 735 740 745
[ [ 2836 ]      pFd-v dKFvD LVTDS PVISA IIDFK TLKNL NDNYG ItRsQ ALdLi RSDPR>
    ^   ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
K8p           705 710 715 720 725 730 735 740 745
[ [ 2735 ]      pFd-M erFnk LiTDS PVISA IIDFK TLKNL NDNYG ItkkQ Amell hSnPk>
    ^   ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^

                                760       770
                                *         *
C486G4p        VLREF INQDN PIIRN RIESL IMQCR L

SA11G4p        755 760 765 770 775
[ [ 3506 ]      VLREF INQDN PIIRN RIESL IMQCR L>
    ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^

RRVVP4p        755 760 765 770 775
[ [ 3198 ]      VLREF INQDN PIIRN RIEqL IMQCR L>
    ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^ ^^^^^
```

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DS-1p      750 755 760 765 770 775
[ 2870 ]   VLRnF INQnN PIIRN RIEqL lIQcK L>
           ^^^ ^ ^^^^^ ^^^^^ ^^^ ^ ^^^^^ ^
M37p       750 755 760 765 770 775
[ 2844 ]   VLRdF INQnN PIIRN RIEqL lIQcK L>
           ^^^^^ ^^^^^ ^^^^^ ^^^ ^ ^^^^^ ^
KUp        750 755 760 765 770 775
[ 2837 ]   VLRnF INQnN PIIRN RIEqL lIQcK L>
           ^^^ ^ ^^^^^ ^^^^^ ^^^ ^ ^^^^^ ^
ST3p       750 755 760 765 770 775
[ 2836 ]   VLRdF INQnN PIIRN RIEqL lIQcK L>
           ^^^^^ ^^^^^ ^^^^^ ^^^ ^ ^^^^^ ^
K8p        750 755 760 765 770 775
[ 2735 ]   tLKEF INnnN PIIRN RIEnL IsQCR L>
           ^^^ ^ ^ ^^^^^ ^^^^^ ^^^ ^ ^^^^^ ^

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Sequence Range: 1 to 2363

10	20	30	40
* GGC TAT AAA ATG GCT TCA CTC ATT TAT AGA CAG TTG CTT ACT AAT	* Met Ala Ser Leu Ile Tyr Arg Gln Leu Thr Asn>	* 90	* 90
50	60	70	80
* TCA TAC ACA GTA GAA CTT TCA GAT GAA ATC CAA GAA ATT GGA TCG	* Ser Tyr Thr Val Glu Leu Ser Asp Glu Ile Gln Glu Ile Gly Ser>	* 130	* 130
100	110	120	130
* ACT AAG ACT CAA AAC GTT ACC GTT AAT CCA GGA CCG TTC GCG CAA	* Thr Lys Thr Gln Asn Val Thr Val Asn Pro Gly Pro Phe Ala Gln>	* 180	* 180
140	150	160	170
* ACA AAT TAC GCT TCA GTT AAT TGG GGA CCT GGT GAA ACG AAT GAC	* Thr Asn Tyr Ala Ser Val Asn Trp Gly Pro Gly Glu Thr Asn Asp>	* 220	* 220
190	200	210	220
* TCA ACT ACA GTT GAA CCA GTG CTT GAT GGA CCA TAT CAA CCA ACG	* Ser Thr Thr Val Glu Pro Val Leu Asp Gly Pro Tyr Gln Pro Thr>	* 270	* 270
230	240	250	260
* ACT TTT AAT CCA CCT GTA AGT TAT TGG ATG TTG TTA GCA CCA ACG	* Thr Phe Asn Pro Pro Val Ser Tyr Trp Met Leu Leu Ala Pro Thr>	* 270	* 270

Figure 4 (Sheet 1 of 9)

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280	290	300	310
AAC GCG GGG GTG GTA GAT CAA GGT ACG AAC AAT ACA AAC AGA TGG			*
Asn Ala Gly Val Val Asp Gln Gly Thr Asn Asn Thr Asn Arg Trp>			
320	330	340	350
TTA GCG ACA ATA TTA ATT AAA CCA AAT GTA CAG CAA GTT GAG CGA		*	*
Leu Ala Thr Ile Leu Ile Lys Pro Asn Val Gln Gln Val Glu Arg>			
370	380	390	400
ACA TAT ACA TTA TTT GGG CAA CAA GGT CAA GTA ACA GTA TCA AAT		*	*
Thr Tyr Thr Leu Phe Gly Gln Gln Val Gln Val Thr Val Ser Asn>			
410	420	430	440
GAT TCA CAG ACA AAG TGG AAG TTT GTG GAT CTA AGT AAG CAG ACA		*	*
Asp Ser Gln Thr Lys Trp Lys Phe Val Asp Leu Ser Lys Gln Thr>			
460	470	480	490
CAA GAT GGT AAT TAT TCA CAA CAC GGT CCT CTA CTG TCA ACA CCG		*	*
Gln Asp Gly Asn Tyr Ser Gln His Gly Pro Leu Leu Ser Thr Pro>			
500	510	520	530
AAA CTG TAT GGA GTG ATG AAA CAT GGA GGT AAA ATT TAC ACT TAT		*	*
Lys Leu Tyr Gly Val Met Lys His Gly Gly Lys Ile Tyr Thr Tyr>			
540			

Figure 4 (Sheet 2 of 9)

SUBSTITUTE SHEET

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550	560	570	580
AAT GGA GAG ACA CCG AAC GCA ACT ACT GGT TAC TAC TCT ACA ACT	*	*	*
Asn Gly Glu Thr Pro Asn Ala Thr Thr Gly Tyr Ser Thr Thr>			
590	600	610	620
AAC TTT GAC ACT GTA AAC ATG ACA GCA TAT TGT GAT TTT TAT ATA	*	*	*
Asn Phe Asp Thr Val Asn Met Thr Ala Tyr Cys Asp Phe Tyr Ile>			
640	650	660	670
ATT CCA TTA GCA CAA GAA GCA AAA TGC ACT GAA TAC ATA AAT AAT	*	*	*
Ile Pro Leu Ala Gln Glu Ala Lys Cys Thr Glu Tyr Ile Asn Asn>			
680	690	700	710
GGA TTA CCA CCA ATA CAA AAT ACG AGA AAT ATC GTA CCA GTT TCG	*	*	*
Gly Leu Pro Pro Ile Gln Asn Thr Arg Asn Ile Val Pro Val Ser>			
730	740	750	760
ATA GTA TCA AGG AAT ATT GTA TAT ACA AGA GCA CAA CCT AAT CAA	*	*	*
Ile Val Ser Arg Asn Ile Val Tyr Thr Arg Ala Gln Pro Asn Gln>			
770	780	790	800
GAC ATA GTG GTA TCA AAA ACT TCA TTA TGG AAA GAG ATG CAA TAT	*	*	*
Asp Ile Val Val Ser Lys Thr Ser Leu Trp Lys Glu Met Gln Tyr>			
810			

Figure 4 (Sheet 3 of 9)

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820	830	840	850
AAT AGA GAT ATA GTG ATA AGA TTT AAA TTT GCT AAC TCA ATC ATA			*
Asn Arg Asp Ile Val Ile Arg Phe Lys Phe Ala Asn Ser Ile Ile>			
860	870	880	890
TCA GGG GGA TTG GGA TAT AAA TGG TCA GAA GTG TCA TTT AAA		*	*
Lys Ser Gly Gly Leu Gly Tyr Lys Trp Ser Glu Val Ser Phe Lys>			
910	920	930	940
GCT AAT TAT CAG TAC ACA TAT ACC AGA GAT GGT GAA GAA GTT		*	*
Pro Ala Asn Tyr Gln Tyr Thr Tyr Thr Arg Asp Gly Glu Val>			
950	960	970	980
GCA CAT ACT ACG TGT TCA GTA AAT GGA ATA AAT GAT TTT AAT		*	*
Thr Ala His Thr Thr Cys Ser Val Asn Gly Ile Asn Asp Phe Asn>			
1000	1010	1020	1030
AAT GGT GGA TCA TTA CCG ACT GAT TTC GTA ATA TCA AAA TAT		*	*
Tyr Asn Gly Gly Ser Leu Pro Thr Asp Phe Val Ile Ser Lys Tyr>			
1040	1050	1060	1070
GTG ATT AAG GAA AAT TCT TTT GTG TAT ATA GAC TAC TGG GAC		*	*
Glu Val Ile Lys Glu Asn Ser Phe Val Tyr Ile Asp Tyr Trp Asp>			

Figure 4 (Sheet 4 of 9)

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1090	1100	1110	1120
* GAT TCA CAA GCA TTT AGA AAC ATG GTA TAT GTA CGC TCG TTG GCA Asp Ser Gln Ala Phe Arg Asn Met Val Tyr Val Arg Ser Leu Ala>	* 1140	* 1150	* 1160
1130	1140	1150	1160
* GCC GAT TTA AAT TCG GTA ATG TGT ACA GGA GGT GAC TAT AGT TTT Ala Asp Leu Asn Ser Val Met Cys Thr Gly Asp Tyr Ser Phe>	* 1180	* 1190	* 1200
1220	1230	1240	1250
* TCA TTG CAT TCA GCA GGT GTA ACT TTA TCA ACG CAG TTT ACA GAT Ser Leu His Ser Ala Gly Val Thr Leu Ser Thr Gln Phe Thr Asp>	* 1270	* 1280	* 1290
1310	1320	1330	1340
* GAA GAA CCG CCG TTC TCA ATT CTA CGG ACC AGA GTT AGT GGA TTG Glu Glu Pro Pro Phe Ser Ile Leu Arg Thr Arg Val Ser Gly Leu>			

Figure 4 (Sheet 5 of 9)

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1360	1370	1380	1390
* TAT GGA CTT CCA GCG GCA AAA CCG AAT AAT TCA CAA GAA TAT TAT Tyr Gly Leu Pro Ala Ala Lys Pro Asn Asn Ser Gln Glu Tyr Tyr>	* 1400	* 1410	* 1420
	* GAG ATA GCT GGG AGA TTT TCA TTA ATA TCA CTC GTA CCG TCA AAT Glu Ile Ala Gly Arg Phe Ser Leu Ile Ser Leu Val Pro Ser Asn>	* 1430	* 1440
	* 1450	* 1460	* 1470
	* GAT GAT TAT CAG ACA CCA ATA ATA AAT TCA GTC ACT GTA CGA CAA Asp Asp Tyr Gln Thr Pro Ile Ile Asn Ser Val Thr Val Arg Gln>	* 1480	
1490	1500	1510	1520
* GAT TTA GAA CGA CAA TTA GGA GAA CTA AGA GAT GAA TTT AAC AAT Asp Leu Glu Arg Gln Leu Gly Glu Leu Arg Asp Glu Phe Asn Asn>	* 1530	* 1540	* 1550
	* TTA TCA CAA CAA ATC GCT ATG TCA CAA CTG ATA GAT CTT GCG TTA Leu Ser Gln Gln Ile Ala Met Ser Gln Leu Ile Asp Leu Ala Leu>	* 1560	* 1570
1580	1590	1600	1610
* CTA CCG TTA GAC ATG TTC TCA ATG TTT TCA GGG ATT AAG AGT ACA Leu Pro Leu Asp Met Phe Ser Met Phe Ser Gly Ile Lys Ser Thr>	* 1620	* 1630	* 1640

Figure 4 (Sheet 6 of 9)

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1900	1910	1920	1930
ACG GAC GGT ATG AAT TTT GAT GAT ATA TCA GCA GCA GTA CTC AAG Thr Asp Gly Met Asn Phe Asp Asp Ile Ser Ala Ala Val Leu Lys>	*	*	*
1940	1950	1960	1970
* ACT AAA ATT GAT AAA TCA ACC CAG TTA AAT ACA AAT ACA TTG CCG Thr Lys Ile Asp Lys Ser Thr Gln Leu Asn Thr Asn Thr Leu Pro>	*	*	*
1990	2000	2010	2020
* GAA ATA GTA ACT GAG GCT TCA GAA AAG TTT ATA CCA AAT AGA GCG Glu Ile Val Thr Glu Ala Ser Glu Lys Phe Ile Pro Asn Arg Ala>	*	*	*
2030	2040	2050	2060
* TAC CGT GTA ATT AAA GAT GAT GAA GTG CTA GAG GCT AGT ACT GAT Tyr Arg Val Ile Lys Asp Asp Glu Val Leu Glu Ala Ser Thr Asp>	*	*	*
2080	2090	2100	2110
* GGT AAA TAT TTC GCT TAC AAA GTT GAA ACC ATT TTG AAG AGA TTC Gly Lys Tyr Phe Ala Tyr Lys Val Glu Thr Ile Leu Lys Arg Phe>	*	*	*
2120	2130	2140	2150
* CAT TCG ATG TAC AAA TTC GCT GAC TTA GTG ACT GAC TCA CCA GTT His Ser Met Tyr Lys Phe Ala Asp Leu Val Thr Asp Ser Pro Val>	*	*	*
2160	2170	2180	2190
*	*	*	*

Figure 4 (Sheet 8 of 9)

# SUBSTITUTE SHEET

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2170      *      2180      *      2190      *      2200      *
ATA TCG GCA ATA ATT GAC TTT AAA ACT CTT AAG AAT CTA AAT GAT
Ile Ser Ala Ile Ile Asp Phe Lys Thr Leu Lys Asn Leu Asn Asp>

2210      *      2220      *      2230      *      2240      *      2250      *
AAT TAC GGA ATA AGC AGA CAA CAA GCA CTA AAT CTT CTA AGA TCT
Asn Tyr Gly Ile Ser Arg Arg Gln Gln Ala Leu Asn Leu Arg Ser>

2260      *      2270      *      2280      *      2290      *
GAT CCG CGA GTA TTA CGT GAA TTT ATT AAT CAG GAT AAT CCA ATA
Asp Pro Arg Val Leu Arg Glu Phe Ile Asn Gln Asp Asn Pro Ile>

2300      *      2310      *      2320      *      2330      *      2340      *
ATA CGA AAT AGA ATA GAA AGT TTG ATA ATG CAA TGT CGC TTG TAA
Ile Arg Asn Arg Ile Glu Ser Leu Ile Met Gln Cys Arg Leu End>

2350      *      2360      *
GCA ACT GAA CAA GAG GAT GTG AC

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Figure 4 (Sheet 9 of 9)

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Sequence Range: 1 to 1062

43/55

10	20	30	40	
* GGC TTT AAA AGC GAG AAT TTC CGT TTG GCT AGC GGT TAG CTC CTT			*	
CCG AAA TTT TCG CTC TTA AAG GCA AAC CGA TCG CCA ATC GAG GAA				Leu Leu>
50	60	70	80	90
* TTA ATG TAT GGT ATT GAA TAT ACC ACA ATT CTA ATC TTC TTG ACA			*	*
AAT TAC ATA CCA TAA TTT ATA TGG TGT TAA GAT TAG AAG AAC TGT				Leu Leu>
Leu Met Tyr Gly Ile Ile Glu Tyr Thr Thr Ile Leu Ile Phe Leu Thr>				
100	110	120	130	
* TCG ATT ACA TTA TTG AAT TAT ATC TTA AAA TCA ATA ACG AGA ATA			*	
AGC TAA TGT AAT AAC TTA ATA TAG AAT TTT AGT TAT TGC TCT TAT				
Ser Ile Thr Leu Leu Leu Asn Tyr Ile Leu Lys Ser Ile Thr Arg Ile>				
140	150	160	170	180
* ATG GAC TAT ATA ATT TAC AGA TTT CTG CTT ATA GTA GTG ATC TTG			*	*
TAC CTG ATA TAT TAA ATG TCT AAA GAC GAA TAT CAT CAC TAG AAC				
Met Asp Tyr Ile Ile Tyr Arg Phe Leu Leu Ile Val Val Ile Leu>				
190	200	210	220	
* GCC ACC ATA ATA AAT GCG CAA AAC TAT GGA GTA AAT TTG CCA ATT			*	
CGG TGG TAT TAT TTA CGC GTT TTG ATA CCF CAT TTA AAC GGT TAA				
Ala Thr Ile Ile Asn Ala Ala Gln Asn Tyr Gly Val Asn Leu Pro Ile>				

Figure 5 (Sheet 1 of 5)

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230 *      240 *      250 *      260 *      270 *
ACA GGT TCA ATG GAT ACT GCG TAT GCA GAC TCT ACA CAA AGT GAG
TGT CCA AGT TAC CTA TGA CGC ATA CGT CTG AGA TGT GTT TCA CTC
Thr Gly Ser Met Asp Thr Ala Tyr Ala Asp Ser Thr Gln Ser Glu>

      280 *      290 *      300 *      310 *
CCA TTT TTG ACA TCA ACC CTT TGT TGT TAT TAT CCT GTT GAG GCA
GGT AAA AAC TGT AGT TGG GAA ACA AAC ATA ATA GGA CAA CTC CGT
Pro Phe Leu Thr Ser Thr Leu Cys Leu Tyr Tyr Pro Val Glu Ala>

320 *      330 *      340 *      350 *      360 *
TCA AAC GAA ATA GCT GAT ACC GAA TGG AAA GAT ACC TTA TCA CAA
AGT TTG CTT TAT CGA CTA TGG CTT ACC TTT CTA TGG AAT AGT GTT
Ser Asn Glu Ile Ala Asp Thr Glu Trp Lys Asp Thr Leu Ser Gln>

      370 *      380 *      390 *      400 *
TTG TTC TTG ACA AAA GGA TGG CCA ACA GGA TCA GTG TAC CTT AAA
AAC AAG AAC TGT TTT CCT ACC GGT TGT CCT AGT CAC ATG GAA TTT
Leu Phe Leu Thr Lys Gly Trp Pro Thr Gly Ser Val Tyr Leu Lys>

410 *      420 *      430 *      440 *      450 *
GAA TAT GCT GAT ATA GCG GCC TTT TCA GTG GAA CCA CAG TTA TAC
CTT ATA CGA CTA TAT CGC CGG AAA AGT CAC CTT GGT GTC AAT ATG
Glu Tyr Ala Asp Ile Ala Ala Phe Ser Val Glu Pro Gln Leu Tyr>

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Figure 5 (Sheet 2 of 5)

SUBSTITUTE SHEET

45/55

460	470	480	490
* TGC GAT TAT AAT TTA GTT TTA ATG AAA TAT GAC TCT ACA CAA GAA			*
* ACG CTA ATA TTA AAT CAA AAT TAC TTT ATA CTG AGA TGT GTT CTT			
* Cys Asp Tyr Asn Leu Val Leu Met Lys Tyr Asp Ser Thr Gln Glu>			
500	510	520	530
* CTA GAT ATG TCT GAA TTG GCC GAT CTT ATA TTG AAC GAA TGG CTG		*	*
* GAT CTA TAC AGA CTT AAC CCG CTA GAA TAT AAC TTG CTT ACC GAC			
* Leu Asp Met Ser Glu Leu Ala Asp Leu Ile Leu Asn Glu Trp Leu>			
550	560	570	580
* TGC AAT CCA ATG GAC ATA ACG CTA TAT TAT TAT CAG CAG ACT GAT		*	*
* ACG TTA GGT TAC CTG TAT TGC GAT ATA ATA ATA GTC GTC TGA CTA			
* Cys Asn Pro Met Asp Ile Thr Leu Tyr Tyr Tyr Gln Gln Thr Asp>			
590	600	610	620
* GAA GCA AAT AAA TCG ATA TGG ACG GGC TCT TCT TGC ACG GTT AAA		*	*
* CTT CGT TTA TTT AGC TAT ACC TGC CCG AGA AGA ACG TGC CAA TTT			
* Glu Ala Asn Lys Ser Ile Trp Thr Gly Ser Ser Cys Thr Val Lys>			
640	650	660	670
* GTG TGT CCA TTA AAT ACA CAA ACA CTT GGT ATT GGA TGT CTA ATA		*	*
* CAC ACA GGT AAT TTA TGT GTT TGT GAA CCA CCA TAA CCT ACA GAT TAT			
* Val Cys Pro Leu Asn Thr Gln Thr Leu Gly Ile Gly Cys Leu Ile>			

Figure 5 (Sheet 3 of 5)

SUBSTITUTE SHEET



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680	*	690	*	700	*	710	*	720	*
ACT AAT CCA GAC ACG TTT GAA ACA GGT GCG ACA ATG GAG AAG TTA									
TGA TTA GGT CTG TGC AAA CTT TGT CAA CGC TGT TAC CTC TTC AAT									
Thr Asn Pro Asp Thr Phe Glu Thr Val Ala Thr Met Glu Lys Leu>									
730	*	740	*	750	*	760	*		
GTG ATT ACA GAT GTT GTA GAT GGT GTC AAT CAC AAA TTA AAC GTC									
CAC TAA TGT CTA CAA CAT CTA CCA CAG TTA GTG TTT AAT TTG CAG									
Val Ile Thr Asp Val Val Asp Gly Val Asn His Lys Leu Asn Val>									
770	*	780	*	790	*	800	*	810	*
ACA ACG GCA ACG TGC ACC ATA CGC AAC TGT AAA AAG TTA GGA CCA									
TGT TGC CGT TGC ACG TGC TAT GCG TTG ACA TTT TTC AAT CCT GGT									
Thr Thr Ala Thr Cys Thr Ile Arg Asn Cys Lys Lys Leu Gly Pro>									
820	*	830	*	840	*	850	*		
AGG GAG AAC GTA GCA GTC ATA CAG GTA GGC GGC GCG AAC ATT TTA									
TCC CTC TTG CAT CGT CAG TAT GTC CAT CCG CCG CGC TTG TAA AAT									
Arg Glu Asn Val Ala Val Ile Gln Val Gly Ala Asn Ile Leu>									
860	*	870	*	880	*	890	*	900	*
GAC ATC ACA GCT GAT CCA ACA ACT ACA CCA CAG ACA GAG ACA ATG									
CTG TAG TGT CGA CTA GGT TGT TGA TGT GGT GTC TGT CTC TGT TAC									
Asp Ile Thr Ala Asp Pro Thr Thr Thr Pro Gln Thr Glu Thr Met>									

Figure 5 (Sheet 4 of 5)

SUBSTITUTE SHEET

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910	920	930	940
ATG CGA ATA AAT TGG AAA AAA TGG TGG CAA GTC TTT TAC ACG GTA	*	*	*
TAC GCT TAT TTA ACC TTT TTT ACC ACC GTT CAG AAA ATG TGC CAT			
Met Arg Ile Asn Trp Lys Lys Trp Trp Gln Val Phe Tyr Thr Val>			
950	960	970	980
GTG GAT TAC GTC AAT CAG ATA ATT CAG ACA ATG TCC AAA AGA TCT	*	*	*
CAC CTA ATG CAG TTA GTC TAT TAA GTC TGT TAC AGG TTT TCT AGA			
Val Asp Tyr Val Asn Gln Ile Ile Gln Thr Met Ser Lys Arg Ser>			
1000	1010	1020	1030
ACA TCG CTT AAT TCG TCG GCG TTC TAC TAT AGA GTG TAG GTG CAT	*	*	*
TGT AGC GAA TTA AGC AGC CGC AAG ATG ATA TCT CAC ATC CAC GTA			
Thr Ser Leu Asn Ser Ser Ala Phe Tyr Tyr Arg Val			
1040	1050	1060	
GCT AGA TTA GAG TTG TAT GAT GTG ACC	*	*	
CGA TCT AAT CTC AAC ATA CTA CAC TGG			

Figure 5 (Sheet 5 of 5)

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	60	70	80	90	100					
Translatio	YRFLl	IVVIL	ATIIN	AQNYG	VNLPI	TGSMD	TAYAD	STQSE	PFLTS	TLCLY
	160	175	190	205	220	235	250	265	280	295
ROTVF7	YRFLf	IiVIL	spflr	AQNYG	iNLPI	aGSMD	TAYAN	STQeE	PFLTS	TLCLY>
[ 1586 ]	^^^^	^^^^	^	^^^^	^^^^	^^^^	^^^^	^^	^^^^	^^^^
	160	175	190	205	220	235	250	265	280	295
RORVP7	YRFLf	IVVIL	splik	AQNYG	iNLPI	TGSMD	TAYAN	STQeE	tFLTS	TLCLY>
[ 1574 ]	^^^^	^^^^	^^	^^^^	^^^^	^^^^	^^^^	^^	^^^^	^^^^
	160	175	190	205	220	235	250	265	280	295
PRVOSUVP7	YRFLl	viVvL	Aplik	AQNYG	iNLPI	TGSMD	TpYmn	SttSE	tFLTS	TLCLY>
[ 1558 ]	^^^^	^^^^	^^	^^^^	^^^^	^^^^	^^	^^	^^^^	^^^^
	160	175	190	205	220	235	250	265	280	295
ROHVP7A	YRFLl	ItVaL	faltr	AQNYG	iNLPI	TGSMD	avYtn	STQeE	vFLTS	TLCLY>
[ 1544 ]	^^^^	^^ ^ ^	^ v	^^^^	^^^^	^^^^	^	^^	^^^^	^^^^
	160	175	190	205	220	235	250	265	280	295
PRVPRVP7G	YRitf	IiVvL	svlsN	AQNYG	iNLPI	TGSMD	TAYAN	STQdn	nFLSS	TLCLY>
[ 1510 ]	^^ v^	^^^^	^ ^	^^^^	^^^^	^^^^	^^^^	^^	^^	^^^^
	890	875								
ROB7	<geyYL	vsl								
[ 53 ]	v	^ ^ ^ ^								
	105									
PRVPRVVP7	1-FLL	IV>								
[ 36 ]	^^	^^								

Figure 6 (Sheet 2 of 7)

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Translatio	110	120	130	140	150
	*	*	*	*	*
YPVEA SNEIA DTEWK DTLsq LFLTK GWPTG SVYLK EYADI AAFSV EPQLY					
ROTVp7	310	325	340	355	370
[ 1586 ]	YPtEA atEin DnsWK DTLsq LFLTK GWPTe SVYfK EYtnI ASFSV dPQLY>				
	^^ ^^	^^ ^^	^^ ^^	^^ ^^	^^ ^^
RORVP7	310	325	340	355	370
[ 1574 ]	YPtEA atEin DnsWK DTLsq LFLTK GWPTG SVYfK EYtDI ASFSV dPQLY>				
	^^ ^^	^^ ^^	^^ ^^	^^ ^^	^^ ^^
PRVOSUVP7	310	325	340	355	370
[ 1558 ]	YPnEA atEIA DTKWt eTLsq LFLTK GWPTG SVYfK gYADI ASFSV EPQLY>				
	^^v^^	^^ ^^	^^ ^^	^^ ^^	^^ ^^
ROHVP7A	310	325	340	355	370
[ 1544 ]	YPtEA StqIn DgdWK DsLSq mFLTK GWPTG SVYfK EYsnI vdfSV dPQLY>				
	^^ ^^	^^ ^^	^^ ^^	^^ ^^	^^ ^^
PRVPRVP7G	310	325	340	355	370
[ 1510 ]	YPpEA ptqIn DnEWK DTLsq LFLTK GWPTG SVYfn EYsnv lefSi dPkLh>				
	^^ ^^	^^ ^^	^^ ^^	^^ ^^	^^ ^^
Translatio	160	170	180	190	200
	*	*	*	*	*
CDYNL VLMKY DSTQE LDMSE LADLI LNEWL CNPMD ITLYY YQQTd EANKS					

Figure 6 (Sheet 3 of 7)

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[illegible]

Figure 6 (Sheet 4 of 7)

# SUBSTITUTE SHEET



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PRVPRVP7G [ [ 1510 ]	760 HKldv ^ ^ ^	775 TstTc ^ ^	790 TIRNC ^ ^ ^	805 nKlGP ^ ^ ^	820 REnVA ^ ^ ^	835 iIQVG ^ ^ ^	850 GSNiL ^ ^ ^	865 DiTAN ^ ^ ^	880 PTTsP ^ ^ ^	895 QTErM> ^ ^ ^
Translatio	310 MRINW	* KKWwQ	VFYTV	320 VDYVN	* QIIQT	330 MSKRS	* TSLNS	340 SAFYy	* RV*VH	350 ARLEL
ROTVp7 [ [ 1586 ]	910 MRINW	925 KKWwQ	VFYTV	940 VDYVd	955 QIIQV	970 MSKRS	985 rSLNS	1000 aAFYy	1015 RV*V*	1030 lRLEL> ^ ^ ^
RORVp7 [ [ 1574 ]	910 MRINW	925 KKWwQ	VFYTV	940 VDYVN	955 QIIQa	970 MSKRS	985 rSLNS	1000 aAFYn	1015 Ri*V*	1030 lwiEm> ^ ^ ^
PRVOSUVP7 [ [ 1558 ]	910 MRINW	925 KrWwQ	VFYTi	940 VDYVN	955 QIVQV	970 MSKRS	985 rSLds	1000 aAFYy	1015 RV*iy	1030 lKLEL> ^ ^ ^
ROHVP7A [ [ 1544 ]	910 MRvNW	925 KKWwQ	VFYTi	940 VDYiN	955 QIVQV	970 MSKRS	985 rSLNS	1000 aAFYy	1015 RV-*y	1030 lRLEL> ^ ^ ^
PRVPRVP7G [ [ 1510 ]	910 MRvNW	925 KKWwQ	VFYTV	940 VDYiN	955 QIVQV	970 MSKRS	985 rSLds	1000 SsFYy	1015 RV*iy	1030 pKLEL> ^ ^ ^

Figure 6 (Sheet 6 of 7)

# SUBSTITUTE SHEET.



Translatio	YDVT
ROTVP7	
[ 1586 ]	YDV>
	^^
RORVP7	1060
[ 1574 ]	YDVT>
	^^^
PRVOSUVP7	1060
[ 1558 ]	YDVT>
	^^^
ROHVP7A	
[ 1544 ]	fDVT>
	^^^
PRVPRVP7G	1060
[ 1510 ]	YDVT>
	^^^

SUBSTITUTE SHEET

Figure 6 (Sheet 7 of 7)

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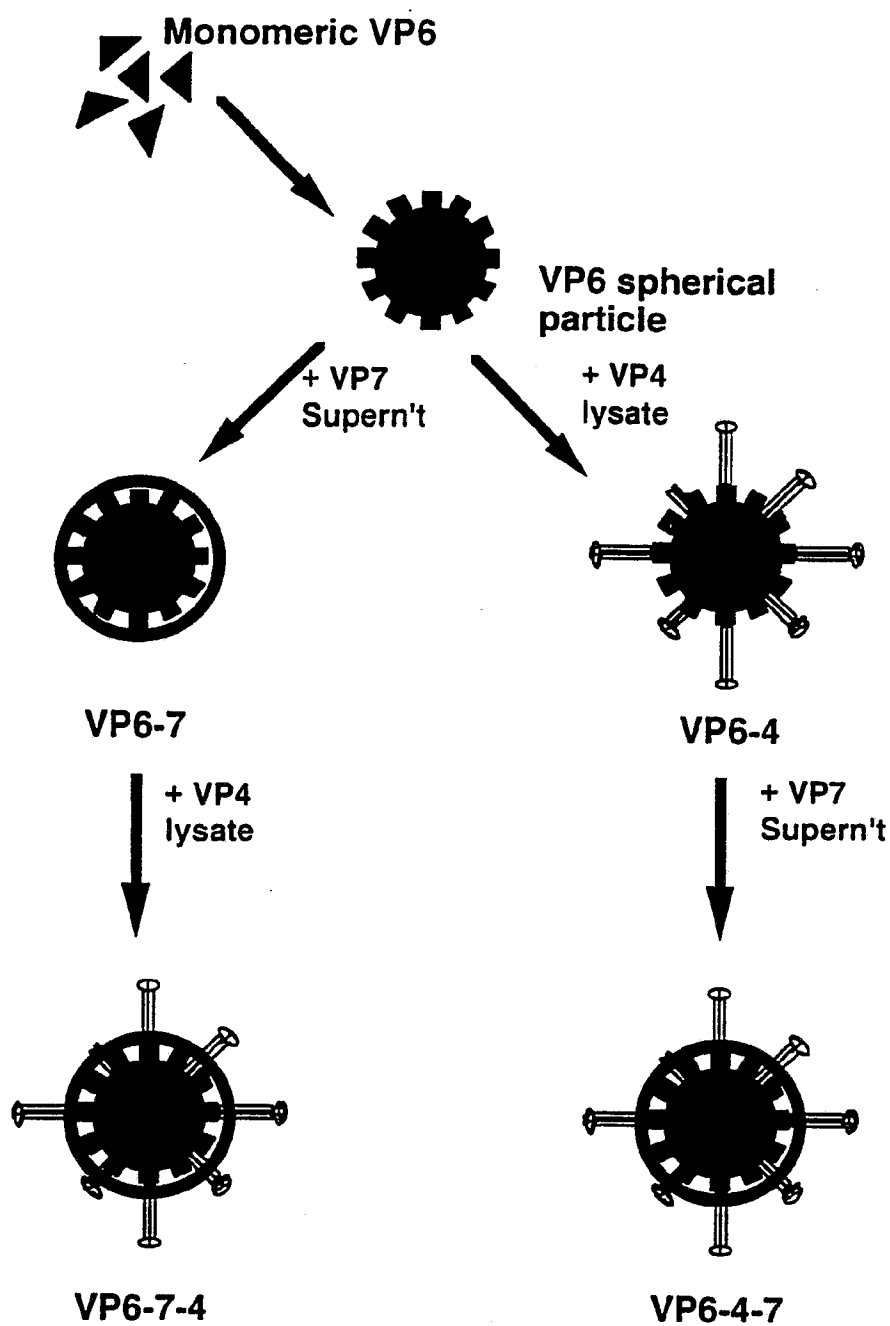


FIGURE 7

SUBSTITUTE SHEET

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 91/00376

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/46 C 12 N 7/04 A 61 K 39/15

**II. FIELDS SEARCHED**Minimum Documentation Searched<sup>7</sup>

Classification System

Classification Symbols

Int.Cl.5

C 12 N

A 61 K

C 07 K

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>**III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>**

Category <sup>10</sup>	Citation of Document. <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Virology, volume 167, no. 1, November 1988, Academic Press, Inc., K.F.M. Ready et al.: "In vitro assembly of the outer capsid of bovine rotavirus is calcium-dependent", pages 269-273, see the whole article (cited in the application) ---	1,3,6, 10
Y	---	8
O,X	Molecular Immunology, volume 28, no. 3, March 1991, Proceedings of the Vaccine Symposium Celebrating the 75th Anniversary of Connaught Laboratories at the University of Toronto, Pergamon Press (GB), M.J. Redmond et al.: "Rotavirus particles function as immunological carriers for the delivery of peptides from infectious agents and endogenous proteins", pages 269-278, see the whole article --- -/-	1,2,5, 10

<sup>10</sup> Special categories of cited documents : <sup>10</sup><sup>"A"</sup> document defining the general state of the art which is not  
considered to be of particular relevance<sup>"E"</sup> earlier document but published on or after the international  
filing date<sup>"L"</sup> document which may throw doubts on priority claim(s) or  
which is cited to establish the publication date of another  
citation or other special reason (as specified)<sup>"O"</sup> document referring to an oral disclosure, use, exhibition or  
other means<sup>"P"</sup> document published prior to the international filing date but  
later than the priority date claimed<sup>"T"</sup> later document published after the international filing date  
or priority date and not in conflict with the application but  
cited to understand the principle or theory underlying the  
invention<sup>"X"</sup> document of particular relevance; the claimed invention  
cannot be considered novel or cannot be considered to  
involve an inventive step<sup>"Y"</sup> document of particular relevance; the claimed invention  
cannot be considered to involve an inventive step when the  
document is combined with one or more other such docu-  
ments, such combination being obvious to a person skilled  
in the art.<sup>"&"</sup> document member of the same patent family**IV. CERTIFICATION**

Date of the Actual Completion of the International Search

16-01-1992

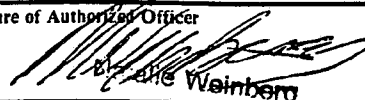
Date of Mailing of this International Search Report

18. 02. 92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorizing Officer



## III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
O,Y	---	8
X	EP,A,0259149 (THE UNIVERSITY OF SASKATCHEWAN) 9 March 1988, see claims	1,2,5,10
Y	---	8
X	Journal of Virology, volume 64, no. 8, August 1990, American Society for Microbiology (US) H. Brüssow et al.: "Polypeptide composition of rotavirus empty capsids and their possible use as a subunit vaccine", pages 3635-3642, see the whole article, especially page 3637, lines 5-12	1-7,9,10,11
Y	---	8
P,X	Journal of Virology, volume 65, no. 6, June 1991, American Society for Microbiology, M.K. Ijaz et al.: "Heterotypic passive protection induced by synthetic peptides corresponding to VP7 and VP4 of bovine rotavirus", pages 3106-3113, see the whole article	1-11,14
Y	EP,A,0273366 (BAYLOR COLLEGE OF MEDICINE) 6 July 1988, see the whole document	8
L	Biosis, BR 41: 19066, see the whole reference (The "L" document is cited to prove that the Symposium mentioned in Mol. Imm. 28, 1991, p 269-278 was held in October 1989)	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers **\*\*** because they relate to subject matter not required to be searched by this Authority, namely:

**\*\* Remark:** Although claims 18-21 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition

2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

CA 9100376  
SA 52346

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 06/02/92. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0259149	09-03-88	AU-B- 608769	18-04-91
		AU-A- 7791887	10-03-88
		JP-A- 63218627	12-09-88
EP-A- 0273366	06-07-88	AU-A- 8310887	30-06-88
		JP-A- 63273478	10-11-88